

Molecular Characterization
of Synapsin Gene
Expression in the Rat

Carola Haas

Disbui. München

1988





Digitized by the Internet Archive
in 2026

https://archive.org/details/IA41555407_0009

Molecular Characterization of Synapsin Gene Expression in the Rat

**Dissertation
zur Erlangung des Doktorgrades der
Fakultät für Biologie
an der
Ludwig-Maximilians-Universität München**

**vorgelegt von
Carola Haas
aus Kempten/Allgäu**

1987

Dissertation eingereicht am: 16.9.87

Sondergutachter: Prof.Dr. L.J. DeGennaro

1. Gutachter: Prof.Dr. C.N. David

2. Gutachter: Prof.Dr. W. Bandlow

Tag der mündlichen Prüfung: 1.2.88

Bob gewidmet

Der vorliegende Bericht wurde vom Verfasser nicht auf
Antrag und unter Aufsicht von Herrn Dr. G. H. H. H. H.
in der Abteilung Biologie des Max-Planck-Instituts für
Physikalische Chemie in Göttingen verfasst.

Die vorliegende Arbeit wurde im Jahr 1934 im Max-Planck-Institut
für Physikalische Chemie in Göttingen verfasst. Der Verfasser
dankt Herrn Dr. G. H. H. H. H. für die Unterstützung und
Bereitstellung der notwendigen Mittel. Die Arbeit wurde im
Jahr 1934 im Max-Planck-Institut für Physikalische Chemie
in Göttingen verfasst. Der Verfasser dankt Herrn Dr. G. H. H. H. H.
für die Unterstützung und Bereitstellung der notwendigen Mittel.

Die vorliegende Arbeit wurde ohne fremde Hilfe auf Anregung und unter Anleitung von Prof.Dr. L.J.DeGennaro in der Abteilung Neurochemie des Max-Planck-Instituts für Psychiatrie durchgeführt.

Prof.Dr. L.J.DeGennaro möchte ich für die Betreuung der Arbeit, seine stetige Hilfsbereitschaft und seine Anregungen danken. Dr. P.Carroll danke ich für die Bereitstellung der genomischen Fragmente des Synapsingens und für hilfreiche Diskussionen. Fr. E.Erhard danke ich herzlich für ihre Kooperation und Hilfe im täglichen Laborablauf.

TABLE OF CONTENTS

I.	SUMMARY.....	1-3
II.	INTRODUCTION.....	4-14
1.	Synapsin I: physicochemical properties and phosphorylation by protein kinase.....	5
2.	Distribution of synapsin.....	6
3.	Interaction of synapsin with the surface of small synaptic vesicles.....	7
4.	Regulation of the state of phosphorylation of synapsin.....	8
5.	Interaction of synapsin with the cytoskeleton.....	9
6.	Phylogenetic aspects of synapsin.....	12
7.	Molecular biological aspects of synapsin.....	12
8.	Aims of this study.....	14
III.	DEVELOPMENTAL REGULATION OF SYNAPSIN GENE EXPRESSION..	15-64
1.	INTRODUCTION OF THE SYSTEM AND THE TECHNIQUES USED..	15-24
1.1	The rat cerebellum as a model system.....	15
1.2	<i>In situ</i> hybridization histochemistry.....	18-22
1.2.1	Parameters of the <i>in situ</i> hybridization.....	20
1.2.1.1	Hybridization conditions.....	20
1.2.1.2	Specificity of <i>in situ</i> hybridization procedure.....	21
1.2.1.3	Choice of radioisotope.....	22
1.3	The SP6 <i>in vitro</i> transcription system.....	23
2.	MATERIAL AND METHODS.....	25-41
2.1	Northern blot analysis.....	25-33
2.1.1	Precautions to avoid RNase contamination.....	25
2.1.2	Isolation of total RNA.....	25
2.1.3.	Purification of poly(A) ⁺ RNA.....	26
2.1.4	RNA denaturation and agarose gel electrophoresis...	27
2.1.5	Northern blot: transfer of denatured RNA to nitrocellulose filter.....	28
2.1.6	Quantitative Northern blot analysis.....	28
2.1.7	Nick-translation.....	30

2.1.8	Determination of incorporated radioactivity by adsorption to DE-81 paper.....	31
2.1.9	Chromatography through Sephadex G-50.....	31
2.1.10	Probes and conditions for hybridization.....	32
2.2	<i>In situ</i> hybridization histochemistry.....	34-41
2.2.1	Coating of glass microscope slides.....	34
2.2.1.1	TESPA-coating.....	34
2.2.1.2	Poly-l-lysine-coating.....	34
2.2.2	Fixation and processing protocols.....	35-37
2.2.2.1	Procedure I.....	35
2.2.2.2	Procedure II.....	35
2.2.2.3	Procedure III.....	36
2.2.3	Hybridization, washing and autoradiography.....	37
2.2.4	Hybridization with ³ H-polyuridyate.....	38
2.2.5	Histological staining.....	38
2.2.6.	Preparation of single-stranded RNA probes for <i>in situ</i> hybridization.....	39
2.2.6.1	Subcloning into pSP65 and propagation in <i>E. coli</i> ...	39
2.2.6.2	Linearization of pSPsyn5 and pSPsyn12.....	39
2.2.6.3	Synthesis of cRNA probes by <i>in vitro</i> transcription.	40
2.2.6.4	Size distribution of <i>in vitro</i> transcription products.....	41
3.	RESULTS.....	42-59
3.1	Northern blot analysis.....	42-49
3.1.1	Accurate size determination of synapsin mRNAs.....	42
3.1.2	Northern blot analysis of synapsin mRNA expression during the development of the cerebellum.....	45
3.1.3	Quantitative Northern blot analysis of the 3.4 kb synapsin mRNA species.....	47
3.2	<i>In situ</i> hybridization histochemistry.....	50-59
3.2.1	Production of synapsin cRNA probes.....	50
3.2.2	Application of <i>in situ</i> hybridization histo- chemistry for the localization of synapsin mRNA in the rat cerebellum.....	53
3.2.3	Localization of synapsin mRNA during the development of the rat cerebellum.....	55
3.2.4	Cellular localization.....	58

4.	DISCUSSION.....	60-64
4.1	Two messenger RNA classes encode synapsin.....	60
4.2	Developmental regulation of synapsin gene expression.....	61
4.3	Correlation of synapsin gene expression and granule cell synaptogenesis.....	62
4.5	Different levels of synapsin gene regulation.....	63
IV.	STRUCTURAL CHARACTERIZATION OF THE SYNAPSIN mRNAs AND THEIR RELATIONSHIP TO THE SYNAPSIN GENE.....	65-100
1.	INTRODUCTION AND GOALS OF THE STUDY.....	65-69
1.1	How are the two synapsin mRNA species related to the synapsin gene ?.....	65
1.2	RNase H assay.....	68
2.	MATERIAL AND METHODS.....	70-73
2.1	Synthesis of oligonucleotides.....	70
2.2	Purification of oligonucleotides by h.p.l.c.....	70
2.3	RNase H digest of RNA/DNA oligomer hybrids.....	71
2.4	Labelling of genomic fragments.....	72
2.5	Probes and conditions for hybridization.....	73
3.	RESULTS.....	74-87
3.1	Hybridization with different synapsin cDNA fragments.....	74
3.2.	Important parameters for the RNase H assay.....	76-77
3.2.1	Nonspecific degradation by contaminating RNases.....	76
3.2.2	Effect of oligonucleotide concentration.....	76
3.3	Mapping of the two synapsin mRNA species by the RNase H assay.....	78-83
3.3.1	Results of cleavage directed by oligo II.....	78
3.3.2	Results of cleavage directed by oligo I.....	82
3.4	Analysis of the structural differences between the two synapsin mRNAs on the genomic level.....	84



4.	DISCUSSION.....	88-100
4.1	Methodological considerations concerning the RNase H approach.....	88-90
4.1.1	Advantages of oligonucleotide-directed cleavage.....	88
4.1.2	Specificity of the RNase H assay.....	88
4.1.3	Effect of secondary structure on RNase H activity.....	89
4.1.4	Resolution of the agarose gel system.....	90
4.1.5	Sensitivity.....	90
4.2.	Structure of the synapsin mRNAs and the processing pathway through which they are generated.....	91-100
4.2.1	The two synapsin mRNA species show a high degree of homology.....	91
4.2.2	The synapsin mRNAs have common 5' but diverging 3' extension.....	92
4.2.3	The synapsin cDNA clone pSyn5 represents a copy of the 3.4 kb mRNA.....	92
4.2.4	Analysis on the genomic level: Identification of an alternative exon.....	93
4.2.5	The 3' end of the synapsin gene is in sight.....	95
4.2.6	An RNA processing model for the synapsin gene.....	97
4.2.7.	Functional aspects of selective synapsin mRNA production.....	100
V.	REFERENCES.....	101-114

List of Abbreviations

ATP	adenosine triphosphate
BRL	Bethesda Research Laboratory
BSA	bovine serum albumin
cdNA	complementary deoxribonucleic acid
cRNA	complementary ribonucleic acid
Ci	Curie
cpm	counts per minute
CsCl	cesium chloride
CTP	cytidine triphosphate
Da	Dalton
DNA	deoxribonucleic acid
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DEP	diethyl pyrocarbonate
dGTP	deoxyguanosine triphosphate
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
E. coli	Escherichia coli
EDTA	disodium ethylene diamine tetraacetate
GTP	guanosine triphosphate
h	hour
h.p.l.c.	high pressure liquid chromatography
kb	kilobases
ml	milliliter
min	minute
MOPS	N-morpholinopropane sulfonic acid
MW	molecular weight
mRNA	messenger ribonucleic acid
NEN	New England Nuclear
nm	nanometer
nt	nucleotides
OD	optical density
p	plasmid
poly(A) ⁺	polyadenylated
PN	postnatal day
PBS	phosphate buffered saline
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
rpm	rotations per minute
RT	room temperature
PBS	phosphate buffered saline
SDS	sodium dodecylsulfate
SEM	standard error of the means
SSC	standard saline citrate buffer
TE	Tris-EDTA-buffer
TEMED	N,N,N,N-tetramethylethylenediamine
TEAA	triethylammonium acetate
TESPA	3-aminopropyl-triethoxysilane
Tris	Tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid
U	unit of enzyme activity
UV	ultra-violet
UTP	uridine triphosphate

I. SUMMARY

As a paradigm for the study of neuron-specific gene expression the regulation of the synapsin gene was investigated during neuronal development. The rat cerebellum was chosen as an *in vivo* model system. Two synapsin mRNAs have been observed in RNA from 10-day-old rat brain by hybridization with a synapsin specific cDNA probe (Kilimann and DeGennaro, 1985). The sizes of these two mRNA species were newly determined to be 3.4 and 4.5 kb. Northern blot analysis was performed using synapsin cDNA as probe to study the expression of the two synapsin mRNAs during neuronal development. It was observed that, in both brain and cerebellum, the appearance of both synapsin mRNAs is differentially regulated. In the cerebellum the 4.5 kb transcript is expressed until postnatal day 7, after which it decreases to an undetectable level. The 3.4 kb mRNA is found throughout cerebellar development and in the adult. The level of this mRNA species was measured by quantitative Northern blot analysis. A peak in the expression of this mRNA was observed at postnatal day 20. This maximum expression of the 3.4 kb transcript coincides with the period of synaptogenesis in the cerebellum.

The spatial distribution of the synapsin mRNAs throughout cerebellar development was determined using *in situ* hybridization histochemistry. An optimized, specific procedure was developed involving hybridization of short ³⁵S-labelled synapsin cRNA probes to Bouin - fixed paraffin sections of the cerebellum. The specificity of the hybridization was proven by pretreatment of sections with RNase A prior to hybridization, and by use of a sense synapsin cRNA as hybridization probe. As a further control, hybridization with ³H-poly(U) detected poly(A⁺) RNA in all cerebellar neurons of all stages studied.

From postnatal day 15 onwards, with a maximum at postnatal day 20, synapsin mRNA was localized in the internal granule cell layer of the cerebellum. On a

cellular level, the granule cells, but not the neighbouring Purkinje cells, express high levels of synapsin mRNA. At this stage of cerebellar development, granule cell axons form synapses with Purkinje cell dendrites. These observations substantiate the correlation of synapsin gene expression with the period of synaptogenic differentiation of neurons.

Taken together, two levels of regulation for the synapsin gene were found through these studies: (i) a developmentally- coordinated decrease in the production of one of the two synapsin mRNAs (ii) the overall levels of expression of the synapsin gene are coordinated with synaptogenesis.

There is a single synapsin gene in rat, mouse and human (Yang-Feng et al., 1986). The two polysome-associated synapsin mRNAs must originate from this gene by some RNA processing mechanism. The first step towards understanding this mechanism was the structural characterization of the two synapsin mRNAs. Through hybridization of different synapsin cDNA fragments to Northern blots of 10-day-old rat brain RNA it was found that both mRNA species share a high degree of sequence homology.

A novel oligonucleotide-directed RNase H assay was elaborated in order to further characterize the structure of the synapsin mRNAs. Oligonucleotides deduced from the synapsin cDNA sequence were used to direct cleavage by RNase H at specific sites within the mRNA molecules. Northern blots of the resulting fragments were hybridized with 3' or 5' specific synapsin cDNA probes respectively. It was found that both synapsin mRNA species have common 5' extension, but diverge in their 3' extensions by about 1000 bases. This result argues for a common transcriptional start site for both mRNAs.

This structural information was a prerequisite to focus the analysis on the genomic level on the 3' portion of the synapsin gene. DNA restriction fragments were chosen from the 3' portion of the synapsin gene and hybridized with Northern blots of poly(A⁺) mRNA from 10-

day-old rat brain. Firstly, the putative 3' end of the synapsin transcription unit could be defined. Secondly, a 1.7 kb Bam HI/Eco RI fragment from the gene recognized the larger 4.5 kb mRNA species with a stronger intensity compared with cDNA fragment E₂. A more refined Northern blot analysis of this area of the gene using smaller genomic restriction fragments revealed gene sequences which almost exclusively hybridized to the 4.5 kb mRNA species. These genomic sequences, hybridizing specifically to the larger mRNA, most probably contain exon(s) which are spliced into the 4.5 kb mRNA species during RNA processing and thus may account for the 1000 base difference between the two mRNAs. These additional sequences are located within or just downstream of the 3' end of the protein-coding region of the 3.4 kb mRNA - whether or not the protein coding sequences are changed awaits DNA sequence analysis.

The results presented here suggest the regulation of synapsin gene expression by alternative splicing of the primary RNA transcript. The evidence argues for a single transcriptional start and 3' polyadenylation site, RNA diversity is created by alternative splicing only.

II. INTRODUCTION

The mammalian nervous system is very complex in its structure and function. It is composed of more than 10^{10} highly specialized nerve cells (see Hubel, 1979), which differ in their anatomical and physiological characteristics. They vary in size, shape, in number of axonal processes that they elaborate and in the number of synaptic contacts that they make. Furthermore, neurons vary in their responsiveness to neurotransmitters and in the type of neurotransmitter that they synthesize and utilize for synaptic transmission. The development of this complicated system in a definite time sequence during ontogenetic differentiation must ultimately be based on the regulation of tissue and cell type-specific gene expression. Therefore a central problem in the study of nervous system development is to discover the molecular mechanisms which act on the level of gene regulation in neurons during such differentiative transitions. Thus far progress towards understanding these mechanisms has been limited.

The enormous complexity of the brain is demonstrated in the recent estimation of the existence of 30000 brain-specific proteins (Bantle and Hahn, 1976; Chikarashi, 1979; Milner and Sutcliffe, 1983). Attempts have been made by to define the genetic regulatory elements which determine neuron-specific gene expression. Sutcliffe et al. (1984) described an 80 base pair sequence (called identifier sequence) in genes destined to be expressed in a neuron-specific fashion. A report of the existence of brain-specific poly(A)⁻ messenger RNA (Van Ness et al., 1979) also tried to demonstrate a motif responsible for neuron-specific gene expression. Both of these ideas have been compromised (Owens et al., 1985; Sapienza and St. Jacques, 1986). A good model system has not yet emerged to allow experimental determination of the elements that influence neuron-specific gene expression.

An alternative approach to this problem begins with

the identification of neuron-specific proteins. A number of nerve-cell specific proteins have been isolated and characterized. Investigation of these proteins with respect to their structure and function has led to insight into nervous system complexity through the study of its individual molecular components. At the level of biochemistry, structure and function one of the best characterized neuron-specific proteins is synapsin I (previously termed protein I). This protein was first identified in 1972 as a major endogenous substrate for cyclic AMP-dependent protein kinase in particulate synaptic fractions of brain (Johnson et al., 1972; Ueda et al., 1973).

1. Synapsin I: physicochemical properties and phosphorylation by protein kinase

Synapsin I is composed of two closely related polypeptides referred to as synapsin Ia and Ib, MW 78 and 74 kd respectively (Ueda and Greengard, 1977; Huttner et al., 1981; McCaffery and DeGennaro, 1986; Schiebler et al., 1986). Both proteins have similar amino acid composition and basic isoelectric points (>10). They both have a relatively high content of proline and glycine and are substrates for degradation by highly purified collagenase. A variety of physicochemical data indicate that both proteins consist of two domains, a globular collagenase resistant domain (head) and an elongated proline-rich domain (tail), which is specifically degraded by highly purified collagenase (Ueda and Greengard, 1977; McCaffery and DeGennaro, 1986; Schiebler et al., 1986).

Synapsin is a physiological substrate for at least three distinct protein kinases in brain and undergoes multisite phosphorylation on serine residues (Krueger et al., 1977; Sieghart et al., 1979; Huttner and Greengard, 1979; Huttner et al., 1981; Kennedy and Greengard, 1981). The head region of synapsin (the collagenase-resistant

domain) can be phosphorylated on a specific serine residue (site 1) with high affinity by either cAMP-dependent protein kinase (Huttner and Greengard, 1979; 1981) or Ca^{2+} /calmodulin-dependent protein kinase I (Kennedy and Greengard, 1981). Synapsin also contains two serine residues (site 2 and 3) in its tail region (collagenase-sensitive domain) that are phosphorylated by Ca^{2+} /calmodulin-dependent protein kinase II (Kennedy et al., 1983; Bennett et al., 1983; McGuinness et al., 1985). Amino acid sequence data, deduced from synapsin-specific cDNA clones, revealed that the collagenase-resistant fragment represents the amino terminal of the protein. The carboxy terminal domain of the protein (the collagenase sensitive portion) contains the two sites for Ca^{2+} /calmodulin-dependent protein kinase II phosphorylation (McCaffery and DeGennaro, 1986).

2. Distribution of synapsin

The distribution of synapsin has been studied with a variety of techniques including radioimmunoassay, sub-cellular fractionation and light and electron microscope immunohistochemistry. Synapsin is present only in the nervous system (Ueda and Greengard, 1977) and within the nervous system it is present only in neurons (Sieghart et al., 1978; De Camilli et al., 1979; Bloom et al., 1979; Fried et al., 1982). Furthermore, synapsin is present throughout both central and peripheral nervous system (DeCamilli et al., 1979; 1983a; Goelz et al., 1981; Fried et al., 1982). Synapsin represents approximately 0.4% of the total protein present in the cerebral cortex of each of five mammalian species studied, indicating that synapsin is a major protein in mammalian brain (Goelz et al., 1981).

Within neurons synapsin has been localized in virtually all nerve terminals of the peripheral and central nervous system, irrespective of their neurotransmitter content (DeCamilli et al., 1983a). On a

subcellular level synapsin has been found to be associated with the outer surface of small (40 - 60 nm) synaptic vesicles (DeCamilli et al., 1983b; Navone et al., 1984).

The association of synapsin with synaptic vesicles in nerve terminals indicates that it is transported from neuronal cell bodies to axon terminals. Evidence for axoplasmic transport of synapsin has come from ligation studies using the bovine splenic nerve. The protein was shown to accumulate mainly proximal to the ligature (Fried et al., 1982).

Synapsin appears in brain during development at about the time of synaptogenesis (Lohmann et al., 1978). Therefore the biosynthesis of synapsin seems to be developmentally regulated.

3. Interaction of synapsin with the surface of small synaptic vesicles

Synapsin is associated with the surface of small synaptic vesicles but it is not an integral membrane protein (Ueda and Greengard, 1977; Huttner et al., 1983;). It can be dissociated from the vesicle by increasing the ionic strength of the suspending medium or by markedly lowering the pH (Ueda and Greengard, 1977; Forn and Greengard, 1978; Huttner et al., 1983). In agreement with these observations, synapsin has been found to be synthesized by free ribosomes (DeGennaro et al., 1983), which synthesize peripheral membrane proteins (Lazarides and Noon, 1984). Binding of synapsin to the vesicle seems to involve the collagenase-sensitive domain of the molecule (Huttner et al., 1983). The binding is specific, saturable, of high affinity and appears to be mediated by a specific protein on the vesicle surface (Huttner et al., 1983; Schiebler et al., 1986). Phosphorylation of the collagenase sensitive domain of the molecule (i.e. at sites 2 and 3) by Ca^{2+} /calmodulin dependent protein kinase II decreases the ability of synapsin to bind to synaptic vesicles (Schiebler et al., 1986).

4. Regulation of the state of phosphorylation of synapsin

A variety of physiological and pharmacological manipulations of intact neurons or nerve endings have been shown to produce changes in the state of phosphorylation of synapsin. A summary of the results of these studies is presented below:

(i) In synaptosomes and in slices of nervous tissue, manipulations that increase the concentration of Ca^{2+} or cAMP inside the axon terminal increase the state of phosphorylation of synapsin (Huttner and Greengard, 1979; Forn and Greengard, 1979; Krueger et al., 1977; Tsou and Greengard, 1982).

(ii) In specific anatomical regions of the central and peripheral nervous system, relevant neurotransmitters (serotonin, dopamine, norepinephrine, adenosine) increase the state of phosphorylation of synapsin (Tsou et al., 1982; Dolphin and Greengard, 1981; Nestler and Greengard, 1980; Mobley and Greengard, 1985).

(iii) In isolated superior cervical ganglion and in posterior pituitary, impulse conduction under physiological conditions increases synapsin phosphorylation (Tsou et al., 1982; Nestler and Greengard, 1982a, 1982b).

(iv) In whole animals, convulsants increase and depressants decrease state of synapsin phosphorylation (Strombom et al., 1979).

In general, manipulations known to induce release of neurotransmitter, or to potentiate release evoked by other stimuli, produce an increase in the ratio of phospho-synapsin to dephospho-synapsin. Most such manipulations are associated with increased levels of cAMP or Ca^{2+} , or both, in the nerve terminal. The sites on the synapsin molecule that become phosphorylated depend upon

the stimulus. When cAMP is involved the head region of the synapsin molecule becomes phosphorylated, whereas when Ca^{2+} is involved, both the head and tail regions become phosphorylated (Huttner et al., 1981; Nestler and Greengard, 1982; Tsou and Greengard, 1982).

These correlations between phosphorylation of synapsin and increased rate of neurotransmitter release, together with the localization of synapsin on synaptic vesicles, suggest that phosphorylation of synapsin may be involved in regulating the release process.

Strong evidence for this function has come from experiments using the squid giant synapse. Injection of the dephospho-synapsin into the presynaptic terminal greatly reduced the amount of neurotransmitter released during a depolarizing step. Conversely, injection of Ca^{2+} /calmodulin dependent protein kinase II potentiated the secretory response to the depolarizing step (Llinas et al., 1985).

5. Interaction of synapsin with the cytoskeleton

The precise function of synapsin is unknown. The high and selective concentration of synapsin on small synaptic vesicles in nerve terminals indicates that it plays a role in some aspect of small synaptic vesicle function. However the lack of synapsin in nonneuronal cells specialized for vesicular secretion, such as exocrine and peptide secreting endocrine cells, indicates that synapsin is not involved in some aspect of the secretory process *per se*, but rather that it plays a role which is specifically related to neuronal secretion.

Small synaptic vesicles undergo local exo-endo-cytotic recycling. This kind of intracellular "traffic" of subcellular organelles is probably regulated by specific interactions with components of the cytoplasmic matrix. Thus interaction of small synaptic vesicle membranes with a specialized cytoskeletal matrix of the nerve terminal seems likely. Recent reports about

interaction of synapsin with cytoskeletal components support this idea. Baines and Bennett (1985) as well as Goodman and Zagon (1986) demonstrated independently that synapsin is immunologically related to the erythrocyte cytoskeletal protein 4.1 and is also a spectrin-binding protein. Recently, synapsin has been shown to interact *in vitro* with neurofilaments (Goldenring *et al.*, 1986) and microtubules (Baines and Bennett, 1986). Finally Bähler and Greengard (1987) described *in vitro* an actin-bundling activity associated with synapsin. This activity is efficiently expressed only by the dephosphorylated form of synapsin. They suggest that upon nerve depolarization, which causes synapsin to be phosphorylated, the bundling activity might be lost.

These data favour the idea that synapsin could be viewed as forming a physical bridge between synaptic vesicles and the cytoskeleton, with the integrity of the bridge regulated by the multisite phosphorylation of synapsin (see fig.1). This idea represents one molecular mechanism through which synapsin might regulate synaptic vesicle availability and neurotransmitter release.

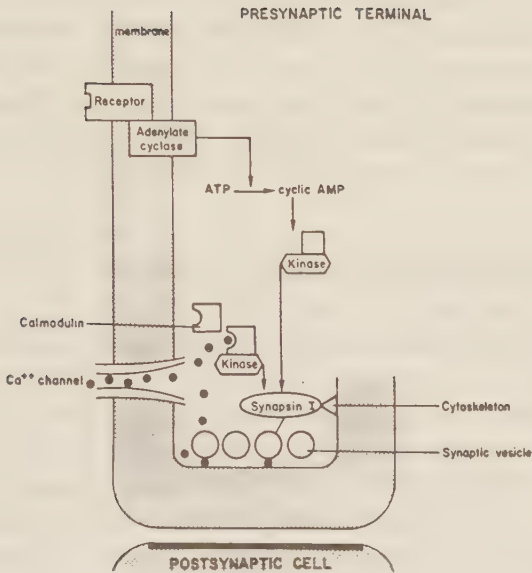


Fig. 1. Diagrammatic representation of the subcellular location and molecular interactions of the synapsin molecule in the presynaptic nerve terminal.

The two protein kinase systems are shown which phosphorylate synapsin: (i) The receptor-mediated adenylate cyclase system which activates the cAMP-dependent protein kinase; (ii) The Ca²⁺-dependent protein kinase system which is activated by Ca²⁺ influx. The synapsin molecule is hypothetically shown as a bridge between cytoskeletal components and synaptic vesicles. The bridging function is indicated to be influenced by phosphorylation through one or the other kinase system.

6. Phylogenetic aspects of synapsin

Phylogenetic studies of synapsin revealed that it is a highly conserved protein. The synapsin molecules in brains of several mammalian species, including human, are immunologically indistinguishable and exhibit similar biochemical and physicochemical properties (Goelz et al., 1981). In addition, proteins that cross-react immunologically with mammalian synapsin have been identified in the nervous system of all other vertebrate classes and three invertebrate phyla (echinodermata, arthropoda and mollusca) (Sorensen and Babitch, 1984; Goelz et al., 1985; Llinas et al., 1985).

7. Molecular biological aspects of synapsin

The evidence described so far suggest that synapsin may play an important role in neuronal function. Therefore it appears to be a prime candidate for a model to study neuronal gene expression. The first step in this approach was the identification of synapsin cDNA clones complementary to synapsin mRNA (Kilimann and DeGennaro, 1985). The availability of these clones enabled the identification of two synapsin mRNA species (Kilimann and DeGennaro, 1985) and the elucidation of synapsin Ib primary structure (McCaffery and DeGennaro, 1986). Through Southern blot experiments, using the synapsin cDNA as probe, a single synapsin gene has been identified in rat (Carroll and DeGennaro, unpublished observation), mouse and human (Yang-Feng et al., 1986). Moreover, the synapsin gene was localized by *in situ* hybridization with synapsin cDNA to the human X-chromosome at band Xp11 (Yang-Feng et al., 1986).

This finding is especially interesting with regard to the fact that nearly 20 human X-linked disorders have been observed that involve abnormal development or function of the nervous system (de Martinville, 1982).

The localization of the synapsin gene to the human X chromosome will be useful in determining whether a mutation in this gene could be responsible for any of these disorders. Disease loci which have been mapped to the region that includes band Xp11 are considered as candidates for possible mutations within the synapsin gene. The gene loci for incontinentia pigmenti (IP), an X-linked dominant disorder prenatally lethal in the male, which has wide-spread effects in the nervous system, has been assigned to Xp11 (Gilgenkranz et al., 1985; Hodgson et al., 1985). Moreover the locus for Norrie's disease, an X-linked recessive disorder which causes blindness, has been mapped to Xp11.3 (Gal et al., 1985; Goodfellow, 1985).

In order to investigate whether a mutation in the synapsin gene could be involved in the diseases described above, or in others effecting the nervous system, detailed knowledge about the organization of the synapsin gene is necessary.

8. Aims of this study

From the existing knowledge about synapsin at the protein, mRNA and gene level a number of interesting questions arise:

Are the two synapsin proteins individually encoded by the two synapsin mRNAs? How are the two synapsin mRNAs regulated during development - do both follow the same pattern of expression? How is their expression correlated with particular events during neuronal development e.g. neurite outgrowth, migration or synaptogenesis? Can changes in synapsin mRNA expression be localized to particular neuronal cell types?

These questions were addressed in the first part of the study described here by Northern blot analysis and *in situ* hybridization histochemistry. The rat cerebellum was chosen as the model system.

The second area of investigation concerned the question of the structural relationship between the two synapsin mRNA species and the single synapsin gene.

What are the structural differences between the two mRNAs? By what mechanism do they originate from the synapsin gene and what are the patterns of transcriptional regulation like?

III. DEVELOPMENTAL REGULATION OF SYNAPSIN GENE EXPRESSION

1. INTRODUCTION TO THE SYSTEM AND TECHNIQUES APPLIED

1.1 The rat cerebellum as a model system

The cerebellar cortex is a simple and uniform structure consisting of three layers that contain only five types of neurons: stellate, basket, Purkinje, Golgi and granule cells. The *molecular layer* is outermost and is composed primarily of granule cell axons, known as parallel fibers, that run parallel to the long axis of the folium. It also contains scattered local interneurons, the stellate and basket cells, as well as the dendrites of neurons lying in deeper layers. The *Purkinje cell layer* consists of the large cell bodies of Purkinje neurons. These neurons send their dendrites up into the molecular layer, where they branch extensively (see also fig. 2). Their axons, however, go down through the granule layer into the underlying white matter. The *granule cell layer* is the innermost and contains a vast number of densely packed small neurons, the granule cells; a few larger Golgi cells are found at the outer border of the granule cell layer.

In the rat the cerebellar cortex develops mainly postnatally. Apart from the Purkinje cells, which originate before birth, the interneurons (basket, stellate and granule cells derive from mitotic cells lying on the surface of the cerebellar cortex in the external germinal layer. Earlier in development these cells have migrated from the neuroepithelium to set up this secondary germinal matrix. The postnatal differentiation of these precursors of granule, basket and stellate cells proceed concurrently with the maturation of the prenatally formed Purkinje cells. In the neonatal cerebellum the Purkinje cells are randomly distributed, during the first postnatal week they line up beneath the external granule cell layer and grow out their huge

dendritic tree into the molecular layer during the second and third week. At the same time the differentiation of the interneurons proceeds in the following order: basket cells during the first, stellate cells during the second and granule cells during the third week, accompanied by the migration of these differentiating neurons to their final position within the cortex. The granule cells migrate from the external germinal layer through the developing Purkinje cell layer into the depth of the cortex, setting up the granule cell layer. Preceding migration the granule cells develop their long bipolar axon, which is left behind when migration starts. This future parallel fiber becomes part of the the molecular layer. In the third postnatal week, when the migration process has finished and the layered structure of the cerebellum has formed, the main period of synaptogenesis starts. These events are summarized in a diagrammatic form in figure 2.

Due to its well-characterized postnatal development and simple cytological architecture the rat cerebellum was chosen as an *in vivo* model system for the study of the pattern of expression and regulation of the synapsin gene during development. In this context the idea was to trace back changes in synapsin gene expression to particular cellular events during cerebellar development. These questions were approached by Northern blot analysis complemented by *in situ* hybridization histochemistry.

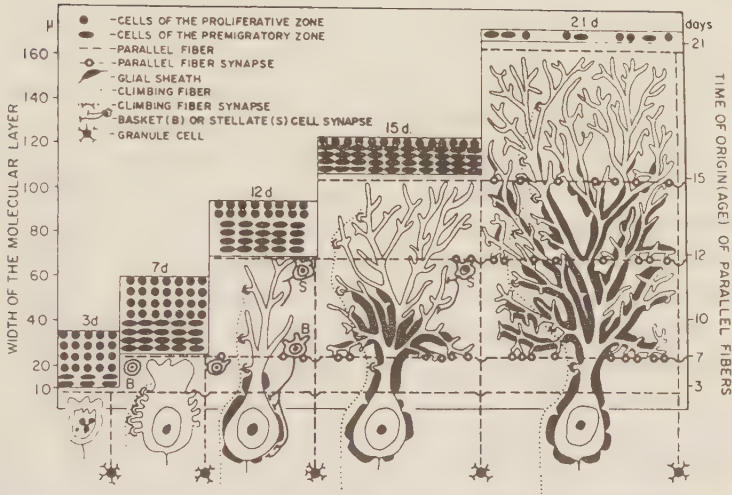


Fig. 2. Diagrammatic illustration of some major events in the maturation of a Purkinje cell and of the molecular layer (adopted from Altman, 1972b).

The width of the molecular layer (left abscissa) as a function of the animals age (5 discrete columns) is based on measurements (Altman, 1972a). Considering the principle of the stacking of parallel fibers from the bottom upward, and disregarding the expansion produced by the invading dendritic processes during synaptogenesis, their location within the molecular layer indicates their "age" or time of origin (right abscissa). If the parallel fibers are traced through the columns from left to right it is seen that the fiber formed on day 7 has no synapses on that day but that it has synapses with basket cells on day 12, and increasing number of synapses from the fiveteenth day onward with dendritic spines of Purkinje cells. On day 21 the fully developed dendritic tree of the Purkinje cell and the parallel fibers of the granule cells are in their main process of synaptogenesis.

1.2 In situ hybridization histochemistry

In situ hybridization is a technique for the localization of specific nucleic acid sequences in individual cells or on chromosomes by hybridization of a complementary DNA or RNA probe with the cellular nucleic acids.

This procedure was originally developed in 1969 by Gall and Pardue, and John et al. for the detection of the genes encoding rRNA in the nucleus of *Xenopus* oocytes. Later it was applied to identify viral genomes in infected cells (Orth et al., 1970; Alonso, 1973; Moar et al., 1975; Singh et al., 1977; Brahic and Haase, 1978). The application of *in situ* hybridization was then extended to the detection of unique genomic sequences in chromosomes (Harper et al., 1981) and to the identification of accumulated globin-specific mRNA in erythroblasts (Harrison et al., 1973; Hudson et al., 1981).

Through the recent advances in recombinant DNA technology complementary DNAs (cDNA) became available as tools to study the expression of cellular genes. Recently *in situ* hybridization histochemistry employing recombinant probes has been applied to localize cellular mRNAs in a variety of tissues (Gee et al., 1983; Jacobs et al., 1983; Uhl et al., 1985; McAllister et al., 1983; Pochet et al., 1983; Cox et al., 1985) or to study the expression of genes regulating embryonal development in *Drosophila* (Hafen et al., 1983, 1984 ; Akam, 1983).

In the brain, with its vast cell type heterogeneity, *in situ* hybridization offers a unique tool for the analysis of function- or development-related changes of gene expression in neuronal subpopulations (Ruppert et al., 1986; Uhl et al., 1985; Gee et al., 1983; Bloch et al., 1985; Griffin et al; 1985; Ginzburg et al., 1986; Wuenschell et al., 1986; Branks and Wilson, 1986; Scott-Young, III et al., 1986; Schalling et al., 1986; Worley et al., 1986).

In situ hybridization histochemistry seemed to be the appropriate approach to study the spatial and tempo-

ral appearance of synapsin mRNA during the development of the rat cerebellum. Through this approach the quantitative Northern blot analysis of changes in synapsin mRNA levels could be complemented by demonstrating the site of synapsin mRNA production in a certain population of neurons.

IN SITU HYBRIDIZATION PROCEDURE

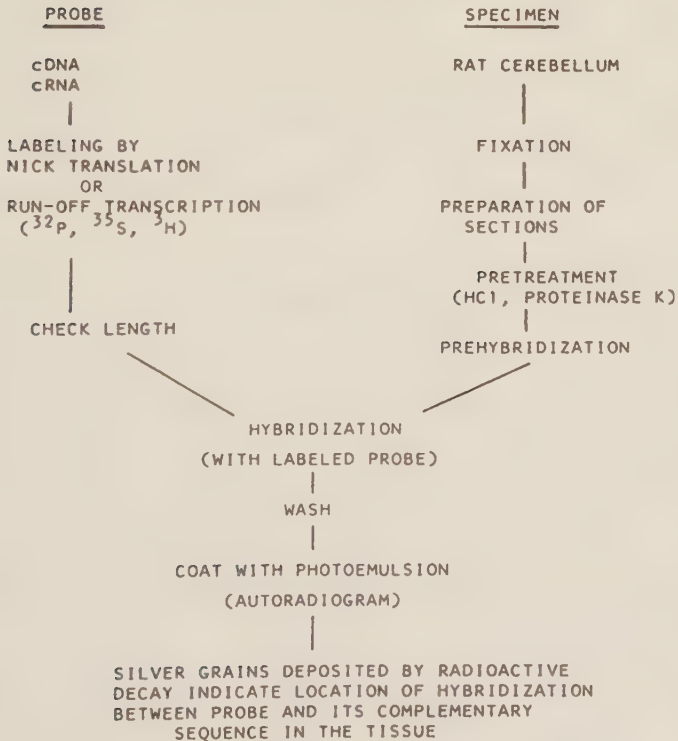


Fig. 3. *In situ* hybridization histochemistry procedure

1.2.1 Parameters of the *in situ* hybridization

1.2.1.1 Hybridization conditions

Success with hybridization *in situ* depends very much on the optimization of the conditions for hybridization and washing. These conditions will vary depending on the tissue and the probe studied. The requirements of any method of hybridization *in situ* include the preservation of the tissue morphology, as well as attainment of optimal specificity and sensitivity.

Sensitivity is dependent upon the preservation of the RNA in the tissue sections and also the preservation of the integrity of the probe and its penetration into the tissue. Thus it is important to inactivate tissue nucleases and prevent nuclease contamination of the sections and the hybridization solution. Fixation of tissue with cross-linking agents probably inactivates tissue nucleases, but for unfixed frozen sections pretreatment with diethyl pyrocarbonate (an RNase inhibitor) is important for inactivation of tissue nucleases (see fixation procedures in Methods).

Hybridization conditions have to be chosen so as to achieve optimal stringency with a minimal background due to non-specific binding of probe and an optimal rate of association of the probe with the tissue mRNA. Important determinants of both stringency and rate of association are the length and concentration of the probe. Short probes (50-200 nt) in high excess diffuse and penetrate better into the tissue to hybridize with the target mRNA. Pretreatment of the tissue with hydrochloric acid and/or protease digestion enhance penetration of the probe.

The stringency of the hybridization is determined by temperature, salt and formamide concentration. The temperature at which 50% of the nucleic acid hybrids melt is known as the melting temperature (T_m). For hybrids *in situ* the T_m is similar to that of filter-bound hybrids (Brahic and Haase, 1978; Cox et al., 1985). Optimal hybridization temperature is considered to be 25°C below

T_m . Formamide decreases the T_m of nucleic acid hybrids and thus allows the hybridization experiment to be carried out at a lower temperature while still preserving stringency. Lower temperatures help preserve tissue morphology and minimize degradation of the probe and tissue RNA.

1.2.1.2 Specificity of *in situ* hybridization

Ascertainment of hybridization specificity is the key to the validation of the *in situ* hybridization procedure. Specificity assessment *in situ* is especially important since tissue sections provide many more opportunities for non-specific interactions with probe than solution hybridization or Northern blot studies, where different RNA species are separated based on their mobilities through agarose gels. The tests of hybridization specificity can be divided in two categories:

- i) **External validity:** tests in this category allow a correlation of results obtained by *in situ* hybridization with data achieved using other techniques i.e. immunohistochemistry or Northern blot analysis.
- ii) **Internal validity:** several criteria for hybridization specificity can be applied within the technique itself. Since the hybridization target is RNA, pretreatment of the tissue of interest with RNase A that digests the RNA, should eliminate hybridization. Although this test is used to support hybridization specificity, it does not provide evidence against the possibility that the probe hybridizes with a related but incorrect RNA sequence. A more specific and reliable control is the use of non-related DNA such as pBR322 plasmid vector or a "sense" cDNA/cRNA of similar length as the specific, complementary probe.

1.2.1.3 Choice of radioisotope

Radiolabelled nucleotides containing tritium, ^{35}S and ^{32}P have been commonly used for *in situ* hybridization when the subsequent analysis is by light microscope autoradiography. The advantage of ^{32}P is that emitted beta particles have a high maximum energy (17 MeV). Results can therefore be obtained after only minimal exposure times. However the transit distance of ^{32}P is a disadvantage since this causes scattering in the signal, which results in a loss of resolution. Probes that contain tritium, a much weaker energy isotope (0.018 MeV), require exposure times of weeks to months, but the lower-energy β -particle of tritium gives probe labelled with this isotope a significant advantage with regard to scatter over ^{32}P -labelled probes, since the trajectory of the β -particle is short.

^{35}S -labelled probes emit a more energetic β -particle than tritium (0.167 MeV) and therefore require less exposure time, while retaining fair cellular resolution. One disadvantage to probes labelled with this radioisotope is an increased level of non-specific binding, perhaps owing to chemical interaction of the reactive sulphur with various tissue components. Addition of high concentrations of DTT or β -mercaptoethanol and cold α -thionucleotide in the hybridization buffer helps to reduce this phenomenon (Shivers *et al.*, 1986; Bandtlow *et al.*, 1987).

1.4 The SP6 *in vitro* transcription system

The SP6-system exploits RNA synthesis by bacteriophage SP6 RNA polymerase, which initiates transcription exclusively at the SP6 promotor, for the synthesis of single-stranded complementary RNA (cRNA) (Butler and Chamberlin, 1982; Kassavetis et al., 1982). Melton et al. (1984) have constructed SP6 cloning vectors that contain an SP6 promotor immediately upstream from a multiple cloning site which allows the insertion of foreign DNA. The recombinant pSP6 vector can be linearized within or downstream of the inserted sequence to generate a template from which the polymerase will "run off" at the end, providing transcripts of any desired length and preventing transcription of any vector sequence (fig. 4 and 10).

RNA transcripts synthesized *in vitro* have been used for different purposes such as hybridization probes (Zinn et al., 1983; Cox et al., 1985) and as substrates for RNA processing studies (Green et al., 1984; Krieg and Melton, 1984). RNA probes offer several advantages compared with traditional nick-translated or end-labelled DNA probes and have been shown to offer important benefits when used for *in situ* hybridization (Cox et al., 1985). RNA probes can be produced in high amounts and with a very high specific activity. They provide a significant increase in hybridization efficiency, since they are single-stranded and short in length. Moreover, the increased thermal stability of RNA/RNA duplexes permits posthybridization washes at higher temperatures and treatment with RNase A, both of which help to reduce nonspecific background. Furthermore, when the cDNA clone of interest is inserted into the SP6 vector in opposite orientation, synthesis yields RNA transcripts whose sequence is identical to the target mRNA. These transcripts provide the most appropriate probe for controls of the *in situ* hybridization procedure.

For these reasons the SP6-system was employed to

produce synapsin-specific cRNA probes to analyze the temporal and spatial distribution of synapsin mRNA expression during the development of the rat cerebellum.

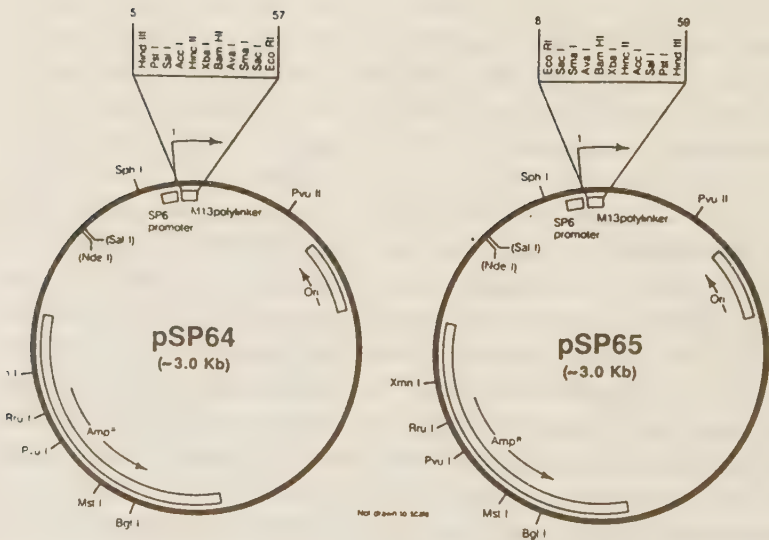


Fig. 4. SP6 transcription vectors, pSP64 and pSP65.

They are constructs derived from pUC12, pBR322 and SP6 DNA. They contain an SP6 promoter immediately upstream from a M13 polylinker sequence. pSP64 and pSP65 differ only in the orientation of the polylinker.

2. MATERIAL AND METHODS

2.1 Northern blot analysis

2.1.1 Precautions to avoid RNase contamination

It is crucial for experiments dealing with RNA to eliminate any contamination with RNases. The following precautions have to be taken:

Disposable gloves should be worn during the experiments. Glassware is usually treated with heat (250°C) for at least four hours to inactivate any residual RNases. Microfuge tubes and pipette tips should be autoclaved. All solutions should be prepared using baked glassware and glass-distilled, autoclaved water. If possible solutions should be treated with 0.1% diethyl pyrocarbonate (DEP), a compound which inactivates RNases, for at least 12 h. Subsequently the DEP should be removed by autoclaving in liquid cycle for at least 15 min. DEP cannot be used in solutions containing Tris, because it destroys the buffering capacity of Tris by covalent binding to the Tris base.

2.1.2 Isolation of total RNA

Precautions were taken to avoid RNase contamination of the preparation as described. Total RNA was isolated from brain and cerebella of rats of various ages using a guanidinium/ cesium chloride method (Glisin et al., 1974; Ullrich et al., 1977). Immediately after sacrifice of the animals brains and cerebella were removed and homogenized in 5 volumes of denaturing solution containing 6 M guanidinium isothiocyanate, 5 mM sodium citrate, pH 7.0, 0.1 M β -mercaptoethanol and 0.5% sarcosyl. After addition of 1 g cesium chloride per 2.5 ml homogenate, the mixture was layered onto a 1.2 ml cushion of 5.7 M CsCl in 0.1 M EDTA, pH 7.5, in Beckman SW 41 polyallomer tubes. The RNA was pelleted by ultracentrifugation at 35000 rpm for 12 h

at 20°C. The pellet was resuspended in 1.5 ml of 10 mM Tris/HCl, pH 7.0, 5 mM EDTA, 1% SDS. In order to improve the dissolution of the RNA pellet in the buffer, the samples were heated for 15 min at 37°C. The dissolved RNA was transferred to a corex tube, extracted with an equal volume of chloroform/butanol (4:1) and centrifuged for 5 min at 10000 rpm in HB 4 rotor to separate the phases. After transfer of the aqueous phase to a new corex tube the organic phase was reextracted with 1.5 ml of the resuspension buffer, centrifuged again and the two aqueous phases combined. Finally the RNA was precipitated by the addition of 0.1 volume of 3 M sodium acetate, pH 5.2, and 2.2 volumes of 95% ethanol and storage at -20°C overnight. After centrifugation at 12000 rpm the RNA pellet was washed with 70% ethanol, spun again and finally dissolved in 0.5 ml 10 mM Tris/HCl, pH 8.0, 1 mM EDTA. Samples were diluted 1:100 for the determination of the optical density at 260 nm. Total recovery of a typical preparation was 0.5 µg RNA/mg tissue. After a second ethanol precipitation the RNA was dissolved in H₂O at 1 mg/ml for storage at -70°C until further use.

2.1.3 Purification of poly(A)⁺ RNA

Poly(A)⁺ RNA was isolated from total brain RNA by oligo(dT)-cellulose chromatography according to Aviv and Leder (1972): (Precautions were taken to avoid RNase contamination as described above). 0.12 milligrams of oligo(dT)-cellulose type 3 (Collaborative Research) were resuspended in 2-3 ml of "low salt" buffer containing 10 mM Tris/HCl, pH 7.5, 1 mM EDTA, 0.05% SDS and poured into a sterile, disposable plastic column. The oligo(dT)-cellulose column was then equilibrated with 5 column bed volumes of "high salt" buffer containing 20 mM Tris/HCl, pH 7.6, 0.5 M NaCl, 1 mM EDTA, 0.1% SDS. Ten milligrams of total RNA from rat brain were dissolved in 2 ml water and heated to 65°C for 5 min, quickly cooled on ice, then warmed to room temperature before the addition of 2 ml of

2 x "high salt" buffer. The RNA was applied to the column, the flow-through collected, heated to 65°C and reappplied to the oligo(dT)-column. After washing with 5-10 column volumes of "high salt" buffer, the poly(A)⁺ RNA was eluted with 2-3 column volumes of "low salt" buffer. The RNA was further purified by a second passage over the oligo(dT)-column as described above. The poly(A)⁺ RNA was precipitated by the addition of 3 M sodium acetate, pH 5.2, to a final concentration of 0.3 M and 2.2 volumes of 95% ethanol at -20°C overnight. After centrifugation (15000 rpm, 30 min, 0°C) the RNA pellet was washed with 70% ethanol and dissolved in 0.5 ml of 10 mM Tris/HCl, 1 mM EDTA, pH 8.0, for optical density reading. The yield of the preparation was determined by comparing the OD₂₆₀ values of the starting material with the oligo(dT) bound material representing the poly(A)⁺ fraction. The average yield was 2 - 5% (= 200 - 500 µg) of poly(A)⁺ RNA compared with total starting RNA (10 mg) after two passages over the oligo(dT)-column.

2.1.4 RNA denaturation and agarose gel electrophoresis

Total or poly(A)⁺ RNA from rat brain or cerebellum was denatured by glyoxylation prior to gel electrophoresis according to Thomas (1980). 2-4 µg of poly(A)⁺ RNA were incubated in a volume of 8 µl with 1 M deionized glyoxal, 50% dimethyl sulfoxide in 10 mM sodium phosphate buffer, pH 7.0, at 50° C for 1 h. The reaction mixture was cooled on ice and 2 µl of sample buffer containing 50% glycerol, 10 mM sodium phosphate, pH 7.0, 0.4% bromophenol blue were added. The samples were electrophoresed on a horizontal 1.0 or 1.2% agarose gel in 10 mM sodium phosphate buffer, pH 7.0, at 50 V for 2-3 h. Constant recirculation of the buffer was required throughout electrophoresis to maintain a constant pH: this is critical, since the glyoxal readily dissociates from the RNA at a pH of 8.0 or above. 3 µg of glyoxylated RNA molecular weight markers (BRL RNA ladder) were run on the

gel adjacent to the brain RNA samples. After electrophoresis the lane containing the marker was cut off, stained with ethidium bromide (2 µg/ml) for 15 min and photographed. The remainder of the gel was carried through Northern transfer.

2.1.5 Northern blot: transfer of denatured RNA to nitrocellulose filter

The transfer procedure was performed according to Thomas (1980). After electrophoresis the gel was placed with its underside uppermost over two sheets of Whatman 3 MM paper saturated with 20 x SSC (3 M NaCl / 0.3 M trisodium citrate). Nitrocellulose paper was trimmed to the same size as the gel, wetted with H₂O, soaked in 20 x SSC for 5 min and laid over the gel. Two sheets of 3 MM paper with the same dimensions as the nitrocellulose filter were soaked in 20 x SSC and placed on top of the filter. Lastly a stack of paper towels and a glass plate was put on top. During transfer the blotting arrangement was sitting in a tray containing 20 x SSC in order to ensure constant buffer supply. Transfer of RNA was complete after 12 h as confirmed by ethidium bromide staining of the blotted agarose gel. After transfer the nitrocellulose filter was removed from the gel, dried at room temperature and baked for two hours at 80°C *in vacuo* in order to fix the RNA to the nitrocellulose.

2.1.6 Quantitative Northern blot analysis

RNA was denatured by glyoxylation; electrophoresed on a 1% agarose gel and blotted onto nitrocellulose as described. Each gel contained a serial dilution of purified 28S and 18S ribosomal RNA from calf liver (Pharmacia) applied as standards adjacent to cerebellar RNA samples. After hybridization with the synapsin specific probe and autoradiography, the blots were

subsequently hybridized with an 18S ribosomal RNA specific cDNA probe (Grummt *et al.*, 1979) which had been labelled with ^{32}P -dCTP by nick-translation. The autoradiogram of the rRNA dilution series was scanned densitometrically (Beckman DU-8 spectrophotometer) and the peak areas were plotted versus the amount of rRNA loaded to give a standard curve. The actual amount of total cerebellar RNA loaded in each lane could be determined by comparing the densitometrically obtained value of 18S cerebellar rRNA with this standard. The signal obtained with the synapsin specific probe was then corrected relative to the amount of total RNA loaded in each lane. Exposure times of the film were chosen to be within the linear range for densitometric scanning.

In order to confirm the reliability of the quantification based on hybridization with a 18S rRNA specific probe, a second method using ethidium bromide was applied (Eiden *et al.*, 1984; Biguet *et al.*, 1986). Northern gels were run in duplicate with serial dilutions of rRNA standards (see above) and cerebellar total RNA samples. One half of the gel was stained with ethidium bromide (30 $\mu\text{g}/\text{ml}$) for 30 min and washed extensively with 40 mM MOPS (N-morpholinopropane sulfonic acid), pH 7.0, 10 mM sodium acetate, 1 mM EDTA for several hours. The other duplicate of the gel was used for Northern transfer. The negative images of U.V.-transilluminated photographs of the ethidium bromide-stained gel were densi-tometrically scanned. The areas of the peaks from 28S and 18S rRNA bands of the cerebellar samples were compared with those of the serial dilution of standard rRNA. The actual amount of RNA loaded in the cerebellar samples could thus be calculated and used to standardize the synapsin specific signal.

2.1.7 Nick-translation

This labelling procedure uses the activity of *E. coli* DNA polymerase I to add nucleotide residues to the 3' hydroxyl terminus that is created when one strand of a double-stranded DNA molecule is nicked. By virtue of its 5' to 3' exonucleolytic activity the enzyme can remove nucleotides from the 5' side of the nick. The elimination of nucleotides from the 5' side and the sequential addition of nucleotides to the 3' side results in movement of the nick along the DNA. In the presence of highly radioactive α - ^{32}P -labelled nucleoside triphosphates, it is possible to prepare ^{32}P -labelled DNA with a specific activity of 10^8 cpm/ μg (Rigby et al., 1977).

The nick-translation reaction was performed basically as described by Rigby et al. (1977). The reaction volume was typically 20 μl containing 0.5 - 1 μg template DNA (purified DNA restriction fragment or whole plasmid with insert), dATP, dCTP, dTTP, 50 μM each; nick-translation buffer (50 mM Tris/HCl, pH 7.2, 10 mM MgSO_4 , 1 mM DTT, 50 $\mu\text{g/ml}$ BSA); 50 μCi α - ^{32}P -dCTP (NEN, 800 Ci/mmol). The reaction mixture was chilled on ice; then a 2×10^4 -fold dilution of DNase I, 1 mg/ml, using ice-cold nick-translation buffer containing 50% glycerol was prepared. 1 μl of diluted DNase I (0.05 $\mu\text{g/ml}$) and 0.1 U of *E. coli* polymerase I (Pharmacia, 100 U/ml) were added to start the reaction, which was allowed to proceed at 15°C for 1 h. The reaction was stopped by the addition of 2 μl of 0.5 M EDTA and adjusted to a final volume of 50 μl with TE (10 mM Tris/HCl, pH 8.0, 1 mM EDTA). The amount of incorporated radioactivity was determined by the DE-81 binding assay as described below. The labelled DNA was separated from the unincorporated nucleotides by chromatography through sephadex G-50 (see below).

2.1.8 Determination of incorporated radioactivity by adsorption to DE-81 paper

After the nick-translation reaction had been completed and stopped, the amount of incorporated radioactivity was determined by binding to DE-81 paper as described by Maniatis et al. (1982).

Two 1 μ l aliquots from each reaction were spotted onto individual 1 x 1 cm discs of Whatman DE-81 paper. One of the discs was washed six times for 5 min in 0.5 M Na_2HPO_4 ; twice in water and twice in 95% ethanol, 1 min each. Both filters, the washed and the unwashed, were dried briefly and then counted in Aqualuma scintillation fluid in a liquid scintillation counter. The counts measured on the unwashed filter represented the total radioactivity in the reaction. The washed filter measured the radioactivity incorporated into the DNA. Usually the amount of incorporated radioactivity ranged between 25% and 30% of the total in a nick-translation reaction. The specific activity of a labelled DNA fragment ranged between $0.5 - 1 \times 10^8$ cpm/ μ g template DNA. These values were comparable to those described by Rigby et al. (1977).

2.1.9 Chromatography through sephadex G-50

This procedure was applied to separate nucleic acids, labelled by nick-translation or *in vitro* transcription from unincorporated ^{32}P -dCTP according to Maniatis et al. (1982). Two methods were available: conventional column chromatography or centrifugation through Sephadex G-50 packed in disposable syringes (spin-column method). The latter procedure was used most frequently because it offered the advantage of being less time consuming and providing the labelled DNA in a small volume after the purification.

Sephadex G-50 was prepared as follows: 30 g of

Sephadex G-50 (medium) were slowly added to 250 ml of TE 8.0 (10 mM Tris/ HCL, pH 8.0, 1 mM EDTA) in a 500 ml beaker and left at room temperature overnight. The supernatant was decanted the next day and replaced with an equal volume of TE 8.0. This stock solution was stored at 4°C and could be used for several weeks.

Spin-column procedure: The bottom of a 1 ml disposable syringe was plugged with sterile, siliconized glass wool. Sephadex G-50, equilibrated in TE 8.0, was filled into the syringe to make a 0.9 ml bed volume. The syringe was then centrifuged at 1600 g for 4 min in a bench centrifuge. Sephadex was added after the spin and centrifuged again until the packed volume was 0.9 ml. 0.1 ml STE (10 mM Tris/HCl, pH 8.0, 1 mM EDTA, 0.1 M NaCl) were added and spun as before. The volume of the nick-translated DNA was adjusted to 100 µl with STE, applied to the column and centrifuged at exactly the same speed as above. The effluent containing the labelled DNA was collected in an eppendorf tube as described in Maniatis et al. (1982).

2.1.10 Probes and conditions for hybridization

The probe for the Northern blot experiments was the restriction fragment 5E₂ of the synapsin cDNA clone pSyn5 (Kilimann and DeGennaro, 1985). It was labelled with ³²P-dCTP by nick-translation to a specific activity of 0.5 - 1.0 x 10⁸ cpm/µg of cDNA as described above. The filter was prehybridized for 3 - 5 h at 50°C in a solution containing 50% formamide, 5 x Denhardt's solution, 4 x SSPE (0.6 M NaCl, 40 mM NaH₂PO₄, pH 7.4, 4 mM EDTA), 0.1% SDS and 100 µg/ml sonicated salmon sperm DNA. Hybridization was then carried out overnight at 50°C in the same solution, with the addition of 2-3 x 10⁶ cpm/ml of heat-denatured nick-translated probe. After hybridization the filters were washed 3 x 15 min in 2 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS and once for 15 min in 0.2 x SSC, 0.1% SDS, all at 60°C, before exposure to X-ray film (Kodak).

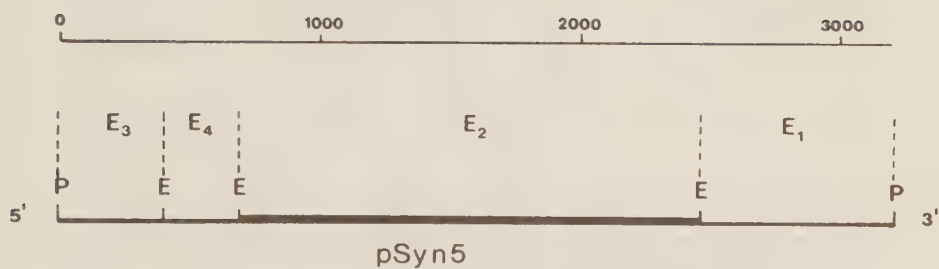


Fig. 5. Incomplete DNA restriction map of the synapsin cDNA clone pSyn5.

Scale at top in base pairs. E₁ - E₄ = designation of Eco R1 restriction fragments of pSyn5. E₂ which is indicated by bold lane, was used as probe in the Northern blot experiments.

2.2 In situ hybridization histochemistry

2.2.1 Coating of glass microscope slides

2.2.1.1 TESPA-coating

Glass microscope slides were thoroughly cleaned in hot soapy water, dehydrated with ethanol and washed twice with acetone. Ordinary coating of the slides with gelatine or poly-l-lysine was not sufficient to allow paraffinized tissue sections to adhere to the glass surface during the hybridization procedure. In order to circumvent this loss of tissue sections, the glass slides were specially treated with TESPA (Fluka, 3-aminopropyl-triethoxysilane) followed by activation with paraformaldehyde (Gottlieb and Glaser, 1975).

The procedure was performed as described by Berger (1986). Cleaned glass slides were submerged in 1% TESPA in toluene for 30 min at 90°C. Thereafter, the slides were washed four times in toluene and air dried. To obtain active aldehyde groups the slides were then incubated in 4% paraformaldehyde, 10 mM K-phosphate, pH 7.0, for 30 min at RT. After washing three times in 10 mM K-phosphate the slides were again air-dried and were ready for use. This treatment allowed covalent linkage of tissue sections to the glass surface and proved to be the best coating procedure for paraffin sections used in the *in situ* hybridization procedure.

2.2.1.2 Poly-l-lysine-coating

In contrast to paraffinized sections, cryostat cut tissue sections adhered to poly-l-lysine-coated microscope slides during the hybridization procedure. Poly-l-lysine coated glass slides were prepared as follows: acid-clean glass microscope slides were immersed in a poly-l-lysine (Sigma, RNase-free) solution containing 50 µg/ml in 10 mM Tris/HCl, pH 8.0, for 30 min at 37°C. The slides were dried in an oven at 37°C overnight and autoclaved.

2.2.2 Tissue fixation and processing protocols

2.2.2.1 Procedure I

This procedure was performed according to McCabe et al. (1986). In order to minimize the chances for degradation of endogenous RNA by RNase in the tissue, rat cerebella were rapidly dissected out of the skull after decapitation and frozen on a cryostat block under high pressure CO₂. Sections of 10 microns were cut on a cryostat, thaw-mounted on poly-l-lysine coated slides and air-dried for 15 min. Subsequently the tissue sections were fixed in 100% ethanol/acetic acid (3:1) for 15 min and immersed in 2 x SSC which had been adjusted to pH 3.5 with acetic acid, for 30 min at 70°C. Deproteination was performed in 2 x SSC/acetic acid, pH 3.5, containing 1 µl/ml Pepsin (Boehringer) for 15 min at 37°C, followed by 1 min rinses in 0.2 x SSC and in water with 5 mM DTT (Dithiothreitol, Sigma). The sections were dried in a vacuum desiccator overnight.

2.2.2.2 Procedure II

The protocol of Shivers et al. (1986) was applied. Cryostat sections were prepared as described above and fixed in 4% paraformaldehyde buffered with PBS, pH 7.0, and 0.02% diethyl pyrocarbonate for 5 min at RT, rinsed once with 0.1 M PBS for 5 min, once with 0.1 M PBS/0.1 M glycine for 5 min and then incubated in 0.3% Triton/PBS for 15 min. Deproteination was carried out with 1 µg/ml Proteinase K (Boehringer) in a solution of 20 mM Tris/HCl, pH 7.5, 2 mM CaCl₂ at 37°C for 15 min. The sections were rinsed with 0.1 M PBS for 3 min, dehydrated in 70%, 80%, 90%, 95% and 100% ethanol and air-dried.

2.2.2.3 Procedure III

Cerebella of young rats of various ages (0, 5, 10, 15, 20, 30 days) were dissected out of the skull and immediately put into Bouin's fixation solution (750 ml picric acid, 250 ml 37% formaldehyde, 50 ml glacial acetic acid) for 4 - 12 h depending on the size of the tissue. The cerebella were cleared of fixative in several changes of 70% ethanol and dehydrated through 80, 90, 95 and 100% ethanol, each for several hours to ensure complete dehydration of the tissue. The cerebella were next soaked in methyl salicylate (Sigma) overnight until the tissue became transparent, followed by three xylene washes, 15 min each.

For complete impregnation with paraffin wax the cerebella were incubated in liquid paraffin overnight at 60°C. The final embedding step was performed on ice which allowed the paraffin to harden quickly in processing cassettes. A needle was used to place the tissue into the desired position within in the block.

Sagittal sections of 10 μ m were cut on a microtome and floated on drops of water placed on acid-clean, TESPA-treated glass microscope slides which were themselves placed on a 37°C heating plate. This step caused the section to stretch out evenly as it dried on the slide. Finally the paraffin sections were fully dried overnight at 37°C in an incubator. They could then be stored for months until further use.

The tissue sections were deparaffinized by three changes (3 x 15 min) in xylene and rehydrated in a descending ethanol series. The pretreatment of the tissue was performed as follows: 10 mM PBS, pH 7.5, rinse for 2 x 5 min; 0.2 N HCl for 10 min at RT; PBS rinse for 2 x 3 min; 1 μ g/ml Proteinase K (Boehringer) in 20 mM Tris/HCl, pH 7.5 and 2 mM CaCl_2 at 37°C for 15 min; PBS with 2 mg/ml glycine, 2 x 3 min; post-fixation with 4% buffered paraformaldehyde for 5 min at RT and a final wash in PBS/glycine for 2 x 3 min. To reduce nonspecific binding,

slices were acetylated by treatment with 0.25% acetic anhydride in 0.1 M triethylamine, pH 7.2, for 10 min at RT (Hayashi et al., 1978), washed in H₂O and dehydrated. Control sections were incubated for 30 min at 37°C with 100 µg/ml RNase A (Boehringer) in 0.5 ml NaCl and 10 mM Tris, pH 7.5.

2.2.3 Hybridization, washing and autoradiography

After pretreatment the dehydrated tissue sections were ready to be hybridized with the synapsin-specific probe. First a prehybridization step was performed to reduce non-specific binding of the probe. Prehybridization (4-6 hours) and hybridization (overnight) were carried out in the following buffer: 50% recrystallized, deionized formamide, 2 x SSC, 1 mM EDTA, 5 x Denhardt's (1 x Denhardt's = 0.02% ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), 0.1 M DTT, 0.5 mg/ml salmon sperm DNA, 0.5 mg/ml heparin, 50 µg/ml tRNA from baker's yeast and 5 µM unlabelled α -thio-UTP at 45°C in a moist chamber. Hybridization was performed in a volume of 8 µl containing 100,000 cpm of ³⁵S-labelled single-stranded cRNA probe (see below). During the hybridization the slices were covered with siliconized, heat-treated coverslips which were sealed with rubber cement (*Fixogum*, Marabu BRD) to prevent evaporation during hybridization.

After careful removal of coverslips, the slides were first washed with 2 x SSC for two hours at 50°C and then with 0.2 x SSC at 50°C until radioactivity was no longer detectable with a Geiger counter. Sections were then treated with RNase A (10 µg/ml) at 37°C for 30 min to remove nonspecifically bound single-stranded RNA probe. Slices were dehydrated through a graded series of ethanol washes containing 0.3 M ammonium acetate; dipped in Kodak NTB-2 photoemulsion (diluted 1:2 in H₂O) (Rogers, 1979); exposed for 4-7 days in a light-proof box at 4°C. They were finally developed in Kodak D 19 Developer for 2 min. After a rinse in tap water the fixation was carried out

in Kodak Fixer for 5 min. Finally the slides were washed for 15 min in tap water and air-dried.

2.2.4 Hybridization with ^3H -polyuridylyate

In order to estimate the poly(A)⁺ RNA content of the cerebellar neurons with respect to increasing age, hybridization with ^3H -polyuridylyate (^3H -poly(U)) was performed. The protocol of Griffin et al. (1985) was applied with slight variations. After fixation and pretreatment the sections were hybridized at 20°C overnight in 10 µl hybridization solution (10 mM Tris/HCl, pH 7.6, 200 mM NaCl, 5 mM MgCl₂, 25% formamide, 500 µg/ml salmon sperm DNA, 100 µg/ml tRNA) containing 40,000 cpm ^3H -poly(U) (3Ci/mmol, NEN). Washing was done in 50 mM Tris/ HCl, pH 7.6, 10 mM KCl, 1 mM MgCl₂ at 50°C for 1 h and at RT for 6 h. The sections were then further processed for autoradiography as described above.

2.2.5 Histological staining

As a last step the sections were incubated in a 0.05% thionin solution for 5 min, rinsed briefly with 70 % ethanol, incubated for 1 min in 95% ethanol, adjusted to pH 5.0 through addition of a few drops of acetic acid, and finally washed thoroughly in 95% and 100% ethanol for 5 min each. The slides were embedded with Entellan (Merck) and examined under the light microscope.

2.2.6 Preparation of single-stranded RNA probes for *in situ* hybridization

2.2.6.1 Subcloning into pSP65 and propagation in *E. coli*

Single-stranded RNA probes were produced by transcription from DNA templates derived by subcloning of the 5E₂ fragment of synapsin cDNA into the plasmid vector pSP65 (Promega Biotec, Madison, WI). The 5E₂ fragment was inserted in opposite orientations with respect to the SP6 promoter, yielding two different subclones pSPsyn5 and pSPsyn12. The orientation of the inserted fragment with respect to the SP6 promoter was checked by restriction digests. Both subclones were propagated in *E. coli* HB 101, large amounts of plasmid DNA were prepared according to the method of Birnboim and Doly (1979) and purified by cesium chloride gradient centrifugation.

2.2.6.2 Linearization of pSPsyn5 and pSPsyn12

Restriction enzyme digest of pSPsyn5 and pSPsyn12 was a requirement to produce linear template DNA of defined length for the *in vitro* transcription with SP6 polymerase.

Purified plasmid DNA (10 µg) were incubated in digestion buffer containing 50 mM Tris/HCl, pH 7.0, 10 mM MgCl₂, 50 mM NaCl, 1 mM DTT in the presence of 5 units of the appropriate restriction enzyme in a total volume of 20 µl. SPsyn5 was digested with *Bgl* II (Boehringer) and SPsyn12 with *Acc* I (Boehringer) for 2 h at 37°C. Test aliquots of the two digested plasmids were run on an agarose minigel to monitor the cleavage. Complete digests were stopped by addition of 2 µl of 0.25 M EDTA, purified by phenol- and ether-extraction and precipitated with ethanol. The linearized plasmid DNA was then dissolved in TE 8.0 and stored in aliquots at -20°C.

The products of the cleavage were as follows: SPsyn5 was cut by *Bgl* II at a unique site in the synapsin cDNA

132 bases distal to the SP6 promotor. This linearized plasmid was expected to yield a transcript of 132 bases (complementary to the synapsin mRNA) in the *in vitro* transcription reaction (Fig. 10; 11). The plasmid SPsyn12 was cleaved by Acc I at two sites within the cDNA and once in the multiple cloning site of the vector, producing fragments of 3100, 1300, 225 bases. The cut of Acc I 129 bases distal to the SP6 promotor yielded the truncated template for the *in vitro* transcription. This transcript (same sequence as the mRNA) had a length of 129 bases (see fig. 10 and 11).

2.2.6.3 Synthesis of cRNA probes by *in vitro* transcription

The reaction was performed in sterile plastic tubes using sterile solutions and sterile DEP-treated water. The transcription reaction was carried out according to the Promega protocol at 37°C for 90 min in a volume of 20 μ l containing:

40 mM Tris/HCl; pH 7.5, 6 mM MgCl₂; 2 mM spermidine (Sigma); 10 mM NaCl; 10 mM DTT; 20 units RNasin ribonuclease inhibitor; 500 μ M each of ATP, GTP and CTP; 0.5 - 1.0 μ g DNA template; 50 μ Ci α -³⁵S-UTP (NEN, 1200 Ci/mmol); 5 - 10 units SP6 polymerase (Promega, Biotec). After completion of the RNA synthesis the template was removed by digestion with 2 units of DNase I (Pharmacia, RNase-free) for 15 min at 37°C in the presence of RNasin (Promega). The *in vitro* synthesized RNA was further purified by phenol extraction and Sephadex G-50 chromatography to remove the unincorporated nucleotides as described. The incorporation of radiolabelled UTP was determined by binding to DE-81 paper as described and reached 80%; this value was in good agreement with that reported by Promega.

2.2.6.4 Size distribution of *in vitro* transcription products

The lengths of the radiolabelled transcription products were examined by separation on an 8% polyacrylamide sequencing gel. The gel was prepared as follows: 15 g urea (Biorad, ultra pure) were dissolved at 37°C in 10 ml distilled water, 6 ml 38% acrylamide and 2% bisacrylamide solution and 3 ml of 1 M Tris-borate buffer, pH 8.3, 20 mM EDTA. This solution was degassed for 5 min. 0.2 ml of 10% ammonium persulfate and 10 μ l TEMED (N,N,N,N-tetramethylethylenediamine, Serva) were added to catalyze polymerization. A 0.3 mm sequencing gel was quickly poured.

Aliquots (ca. 0.5×10^6 cpm) of the radiolabelled transcripts were lyophilized and dissolved in 5 μ l loading buffer (80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue), heated to 90°C for 1 min and quickly cooled on ice. The denatured samples were immediately loaded on the gel with glass capillaries and run at 1200 V in 100 mM Tris-borate buffer, pH 8.3, until the marker dye had run to the bottom of the gel. The gel was then fixed in 10% acetic acid for 10 min; removed from the glass plates; mounted on Whatman 3 MM paper; dried on a gel drier and covered with Saran wrap. The dry gel was then exposed to X-ray film with an intensifying screen at -70°C overnight.

3. RESULTS

3.1 Northern blot analysis

3.1.1 Accurate size determination of the synapsin mRNAs

Previous experiments had demonstrated the existence of two synapsin mRNA species, 4.5 and 5.8 kb in size (Kilimann and DeGennaro, 1985). In order to test the accuracy of this estimation the sizes were re-determined through the application of commercially available RNA size standards, the BRL RNA Ladder. This preparation contains a set of six RNA transcripts (ranging in size from 0.3 - 9.5 kb) which were synthesized *in vitro* by a bacteriophage transcription system. The RNA Ladder provides a set of molecular weight standard for estimating the size of RNA molecules after denaturing agarose gel electrophoresis and subsequent ethidium bromide staining. The transcripts are copies of yeast and lambda DNA sequences and can also be detected on Northern blots by hybridization with radiolabelled lambda DNA probes.

The sizes of the synapsin mRNAs were newly determined by comparison with the migration of the RNA Ladder standards as visualized by both methods: ethidium bromide staining and by hybridization with nick-translated lambda DNA.

Poly(A)⁺ RNA of 10-day-old rat brain and 2 x 3 µg of RNA Ladder were glyoxylated and size-fractionated on a 1.2% agarose gel in adjacent lanes. After electrophoresis one marker lane was cut off and stained with ethidium bromide (10 µl/ml) for 20 min and was photographed next to a ruler (fig. 6, lane 1). The remainder of the gel was blotted onto nitrocellulose. The position of the synapsin mRNA on the blot was determined by hybridization with the nick-translated synapsin cDNA fragment 5E₂ (fig. 6, lane 3) whereas in parallel the RNA size markers were visualized through hybridization with radiolabelled lambda DNA (fig. 6, lane 2). Thereafter the sizes of the

synapsin mRNAs could be determined by comparison of their migration distance with standard curves derived from the ethidium bromide stained or autoradiographically visualized RNA Ladder (fig. 6). Both methods worked equally well and helped to define the accurate sizes of the synapsin mRNAs at 3.4 and 4.5 kb (fig. 6).

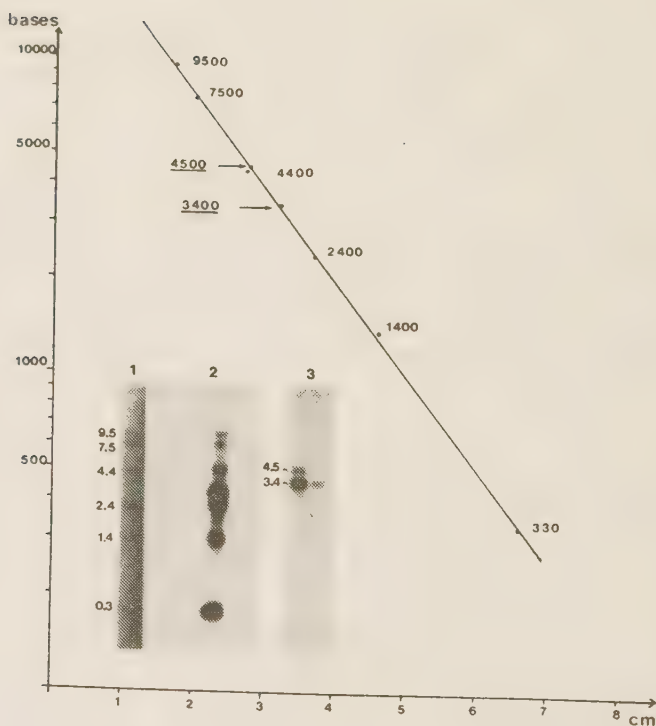


Fig. 6. Size determination of the synapsin mRNAs.

Standard curve established by measuring the migration distance of marker RNAs (0.33 - 9.5 kb) in relation to their molecular weights in a 1.2% agarose gel. Sizes of synapsin mRNAs were determined by comparison of their migration distance with this standard curve (arrows). Lane 1: Size-fractionated BRL RNA Ladder, stained with ethidium bromide. Sizes of RNA Ladder components in kilobases. Lane 2: Northern blot of RNA Ladder detected by hybridization with ^{32}P -nick-translated lambda DNA. Lane 3: Northern blot of poly(A)⁺ RNA from 10-day-old rat brain hybridized with ^{32}P -nicktranslated synapsin cDNA fragment 5E₂.

3.1.2 Northern blot analysis of synapsin mRNA expression during the development of the cerebellum

In order to study synapsin gene expression during cerebellar development Northern blots were carried out using total RNA extracted from rat cerebella of various ages. The blots were hybridized with ^{32}P -labelled 5E₂, a restriction fragment of the synapsin cDNA clone pSyn5 (Kilimann and DeGennaro, 1985), and exposed to X-ray film after washing. The results of these experiments show that the two synapsin transcripts are differentially regulated during the development of the rat cerebellum (fig. 7 A, B). The larger 4.5 kb transcript was easily detectable in cerebellar RNA at birth and through postnatal day 7 (PN 7), it then decreased rapidly to almost undetectable levels through PN 10, 15 and in the adult. The smaller mRNA species (3.4 kb), however, was expressed throughout cerebellar development and in the adult. This phenomenon was not restricted to the cerebellum. Differential regulation of the two mRNA species could also be seen by comparing RNA from 10-day-old and adult rat brain minus cerebellum (fig. 7 B). Furthermore, the 4.5 kb transcript was not detected in RNA from adult rabbit cerebellum (fig. 7 B, lane 4). These data indicate that differential regulation of the synapsin mRNAs occurs during development in both the rat brain and cerebellum and possibly in the cerebellum of at least one other species, the rabbit.

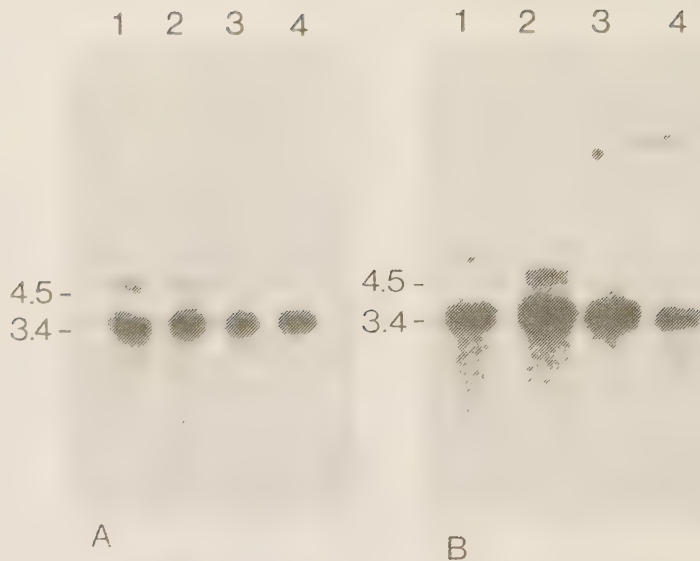


Fig. 7. Differential regulation of synapsin mRNA expression.

Northern blot analysis of rat cerebellar RNA from various ages as described in methods hybridized with ^{32}P -nick-translated synapsin 5E₂ cDNA fragment as probe. Panel A: Total RNA from rat cerebellum of various ages. Lane 1: newborn, lane 2: 7 days, lane 3: 10 days, lane 4: 15 days. (Exposure time: 2 days, at -70°C). Various amounts (1-2 μg) of RNA were used for the different stages to achieve equal intensity for the 3.4 kb band. This allowed a better comparison of the ratio of the 3.4 and 4.5 kb bands during development. Panel B: Northern blot of total RNA hybridized as in Panel A. Lane 1: adult rat brain minus cerebellum, lane 2: 10-day-rat brain minus cerebellum, lane 3: adult rat cerebellum, lane 4: adult rabbit cerebellum.

3.1.3 Quantitative Northern blot analysis of the 3.4 kb synapsin mRNA species

In order to elucidate more precisely, whether or not the levels of the 3.4 kb mRNA species were regulated specifically during postnatal development of the cerebellum a quantitative analysis was performed. The levels of this mRNA were quantified by densitometric scanning of autoradiograms like that shown in figure 8 (see details of quantification procedure in Material and Methods). The 4.5 kb transcript is not visible in this autoradiogram because of the short exposure time chosen to ensure the linearity of the autoradiographic signal. A graphic representation of the standardized data is shown in figure 9. It is evident that the level of the 3.4 kb mRNA species changes during cerebellar development: in contrast to a relatively low level from birth to PN 10, there is a five-fold increase by PN 20. This peak of expression is followed by a decrease to the adult level.

Morphological studies in the rat have shown that the axons of the cerebellar granule cells, the predominant neuronal cell type in the cerebellum, establish their synaptic connections with the dendritic trees of the Purkinje cells between PN 15 and PN 30 (Altman, 1972 a,b). Therefore, the observed peak of synapsin mRNA expression at PN 20 coincides with the period of synaptogenesis of the granule cells.

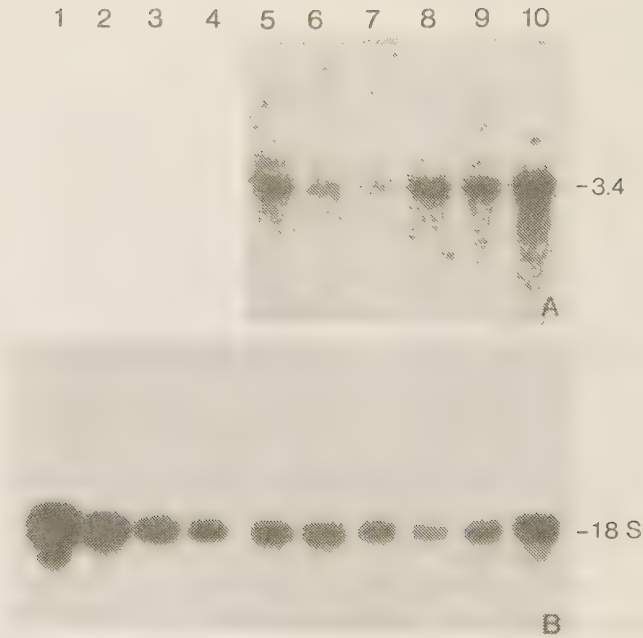


Fig. 8. Quantitative Northern blot analysis of the 3.4 kb synapsin mRNA during rat cerebellar development.

Panel A: Northern blot of total RNA from cerebella of various ages hybridized with ^{32}P -labelled 5E_2 probe. Lane 5: newborn, lane 6: 5 days, lane 7: 10 days, lane 8: 20 days, lane 9: 30 days, lane 10: adult. (Exposure time: 3 days, at -70°C). Panel B: The same blot as in A was subsequently hybridized with a nick-translated 18S rRNA cDNA probe. Lane 1-4: serial dilution of standard calf liver rRNA, 2 μg , 1 μg , 0.5 μg , 0.25 μg . Lane 5-10: as in A. (Exposure time 3 h, at -70°C).

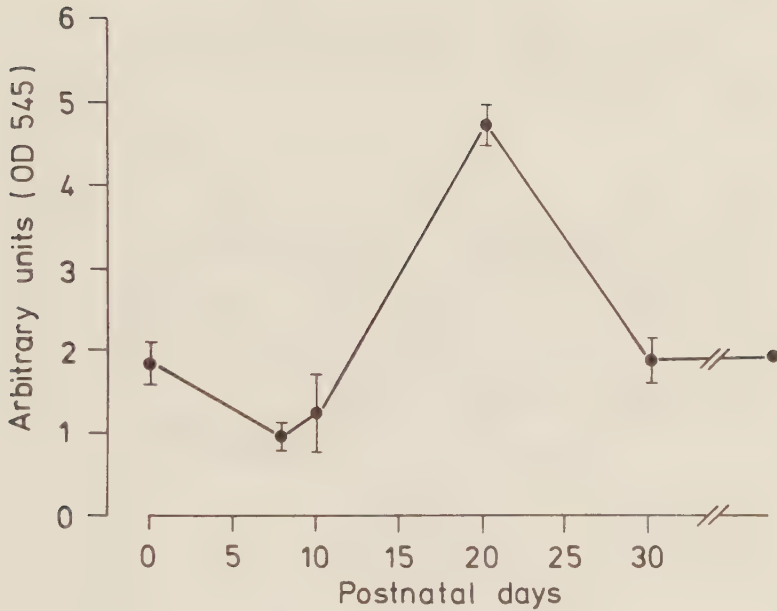


Fig. 9. Quantification of 3.4 kb synapsin mRNA by densitometric scanning of autoradiograms like that shown in Figure 8.

First the autoradiogram of the rRNA dilution series was scanned and the peak areas plotted versus the amount of rRNA, to give a standard curve. Using this standard curve the actual amounts of total cerebellar RNA in each experimental lane were determined. The signal obtained with the synapsin specific probe was then corrected relative to the amount of total RNA loaded in each lane. The corrected values for the synapsin specific signal are shown. The values represent the mean + SEM from at least three independent experiments.

3.2 *In situ* hybridization histochemistry

3.2.1 Production of synapsin cRNA-probes

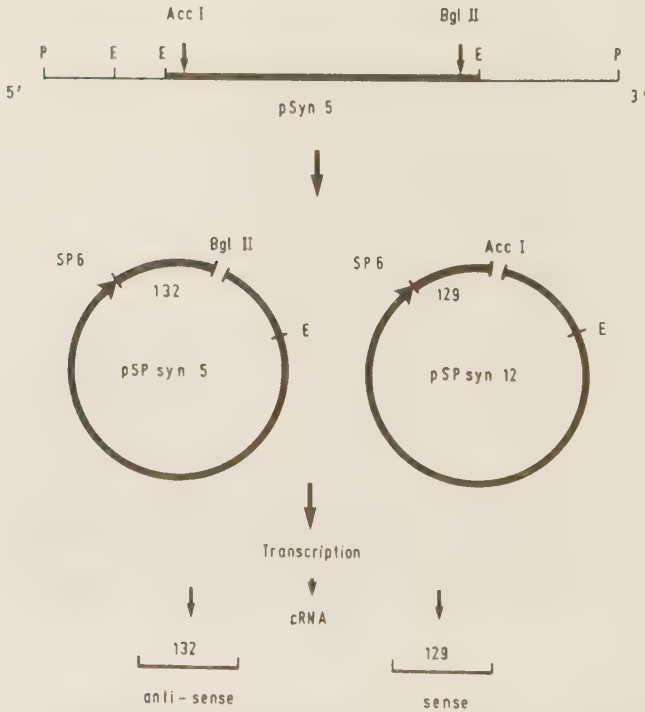


Fig. 10. Production of synapsin cRNA probes using the SP6 in vitro transcription system (see text).

Single-stranded synapsin cRNA probes were produced by *in vitro* transcription from DNA templates derived by subcloning of the 5E₂ fragment of the synapsin cDNA into the plasmid vector pSP65 (Promega Biotec). The two clones used (pSPsyn5 and pSPsyn12) contained the 5E₂ fragment in opposite orientation with respect to the SP6 promotor. Transcription of pSPsyn12 and pSPsyn5 with SP6 polymerase yielded transcripts that were sense (identical to mRNA) and anti-sense (complementary to mRNA). Transcription reactions were performed with Bgl II (anti-sense)- and Acc I (sense)-linearized plasmids in the presence of ³²p- or ³⁵S-UTP, which yielded probes of 132 and 129 nt, respectively (fig. 10 and 11).

The sizes of these transcripts were within the range recommended by Cox et al. (1985) as being optimal for *in situ* hybridization. In addition, the sense transcript derived from pSPsyn12/Acc I template was considered to be an optimal probe for control experiments, because it had the same size as the complementary probe and therefore was directly comparable with regard to hybridization efficiency and penetration kinetics.



Fig. 11. Size determination of ^{32}P -labelled *in vitro* transcription products derived from pSPsyn5 and pSPsyn12.

Aliquots of the individual transcription reactions were size-fractionated on a 8% denaturing urea polyacrylamide gel and visualized by autoradiography. Lane 1: 132 base transcript derived from pSyn12/Bgl II, representing a full-length transcript, no smaller products detectable. Lane 3 and 4: same as in 1, different fractions of Sephadex G-50 column showing only little contamination with free ^{32}P -UTP. Lane 2: 129 base transcript derived from pSyn12/Acc I template, also full-length. Lane 5: Lambda/Hind III cut DNA as MW standard, 125 bp.

3.2.2 Application of *in situ* hybridization histochemistry for the localization of synapsin mRNA in the rat cerebellum

In situ hybridization histochemistry is a powerful and sensitive technique, but it has to be adapted and refined for the individual system one wants to work with.

The first critical step of the procedure is the fixation of the tissue. It should satisfy two main requirements: good tissue preservation and accessibility of the probe to the mRNA in the tissue. In order to find the appropriate fixation method to allow *in situ* hybridization on rat cerebellum, three different fixatives were tested with respect to the following parameters: tissue preservation, RNA retention, probe accessibility and hybridization specificity.

All three methods have been already reported as being suitable fixation techniques for brain tissue: (i) 100% ethanol/acetic acid (3:1) (Ruppert et al., 1986; McCabe et al., 1986); (ii) 4% paraformaldehyde buffered with PBS (Gee and Roberts, 1983; Branks and Wilson, 1986; Shivers et al., 1986; Wuenschell et al., 1986); (iii) Bouin's solution (picric acid, formalaldehyde, acetic acid) (Griffin et al., 1985; Ginzburg et al., 1985; McAllister et al., 1983). The different fixation procedures were tested in combination with cryostat versus paraffin sections of 20-day-old rat cerebella (the stage of maximal synapsin mRNA expression), and different processing protocols (see Methods). Hybridization was carried out with ³²P-labelled cRNA produced by *in vitro* transcription as described.

In cerebellar cryostat sections fixed with ethanol/acetic acid synapsin specific hybridization was observed in the internal granule cell layer, but not in the molecular or the Purkinje cell layer. The control sections which were hybridized with sense-cRNA probe made by *in vitro* transcription as described, or RNase A pretreated sections, showed only background hybridization. Although this fixative appeared to give

reproducible specific synapsin mRNA hybridization, indicating sufficient penetration of the probe, the morphology of the tissue was poorly maintained. The preservation of the tissue was not good enough for cellular localization of synapsin mRNA. This observation was in agreement with Gee and Roberts (1983) and Griffin et al. (1985), who described ethanol/acidic acid fixation as giving reproducible specific results, but poor morphology.

Paraformaldehyde-fixed cryostat sections showed better tissue preservation and also a synapsin-specific signal in the internal granule cell layer, but surprisingly high accumulation of silver grains over white matter fiber tracts indicating completely non-specific binding of the probe. In negative control sections hybridized with the sense cRNA probe some non-specific binding to the internal granule cell layer was observed. Although this fixation medium offered the advantage of reasonable morphology, it carried the drawback of non-specific hybridization.

The third fixative tested, Bouin's solution, gave more promising results. Immersion-fixation in combination with embedding in paraffin for slicing resulted in very good morphology and a synapsin-specific hybridization signal in the internal granule layer.

After comparison of these fixatives in combination with slightly different processing protocols Bouin's fixation appeared to be superior with regard to tissue preservation and hybridization specificity. Hence it was used for the *in situ* hybridization study of synapsin gene expression during the development of the rat cerebellum.

3.2.2 Localization of synapsin mRNA during the development of the rat cerebellum

A developmental time course of synapsin mRNA appearance in the rat cerebellum had been established by quantitative Northern blot analysis. Localization of synapsin mRNA *in situ* provided additional evidence for the temporal and spatial expression of the synapsin gene in the cerebellum. To address the question of temporal expression, *in situ* hybridization using ^{35}S -labelled single-stranded synapsin cRNA probe was carried out on sections of the cerebellum from rats at different developmental stages.

An optimized *in situ* hybridization protocol was applied including the following improvements: paraffin sections of Bouin-fixed cerebella, which provided good morphology and RNA retention (tested with ^3H -poly(U) hybridization); use of a short (132 nt), single-stranded synapsin cRNA probe hybridizing with higher efficiency than a double-stranded probe; labelling with ^{35}S -isotope, which offered high specific activity and good resolution; nonspecific binding associated with the use of this isotope (also described by Shivers *et al.*, 1986; Bandtlow *et al.*, 1987) was inhibited by inclusion of 0.1 M DTT and unlabelled α -thio-UTP in the hybridization buffer and by a post-hybridization treatment with RNase A (Cox *et al.*, 1985). Hybridization background was strongly reduced by acetylation (Hayashi *et al.*, 1978) of the sections and the inclusion of heparin in the hybridization mix (Singh and Jones, 1984). The hybridization was carried out at 45°C, which was 25°C below the calculated T_m of the 132 nt (anti-sense) and 129 nt (sense) cRNA-probes.

Negative controls to demonstrate the specificity of the hybridization sections included predigestion with RNase A prior to hybridization and hybridization of sections with the sense-probe.

The results of the developmental time course of synapsin expression were: In young cerebella (PN 0 and PN 5), no synapsin specific signal was observed (fig. 12 A,

B; 13 B). At this stage of development the layered structure of the cerebellum has not yet formed and the undifferentiated granule cells still reside in the external granule cell layer. From PN 15 onward, however, synapsin-specific expression was detectable in the internal granule cell layer, but not in the external granule cell layer (fig. 12 C). The synapsin specific signal increased sharply at PN 20 in the granule cell layer (fig. 12 E and F). In control experiments in which an RNase A pretreatment of the sections was performed, the signal could be abolished (not shown). Control sections hybridized with the sense strand probe show a few grains homogeneously distributed over all layers of the cerebellum and confirm the specificity of the synapsin cRNA hybridization (fig. 12 D).

The developmental time course of synapsin mRNA expression described by *in situ* hybridization corresponds well with the result of the Northern blot analysis described above. It confirms that the peak of synapsin gene expression is correlated with the synaptogenesis of cerebellar granule cells. Synapsin mRNA in young cerebella (PN 0, 5, 10), demonstrated by Northern blot analysis, was not visualized *in situ*. This suggests that it lies below the present level of detection.

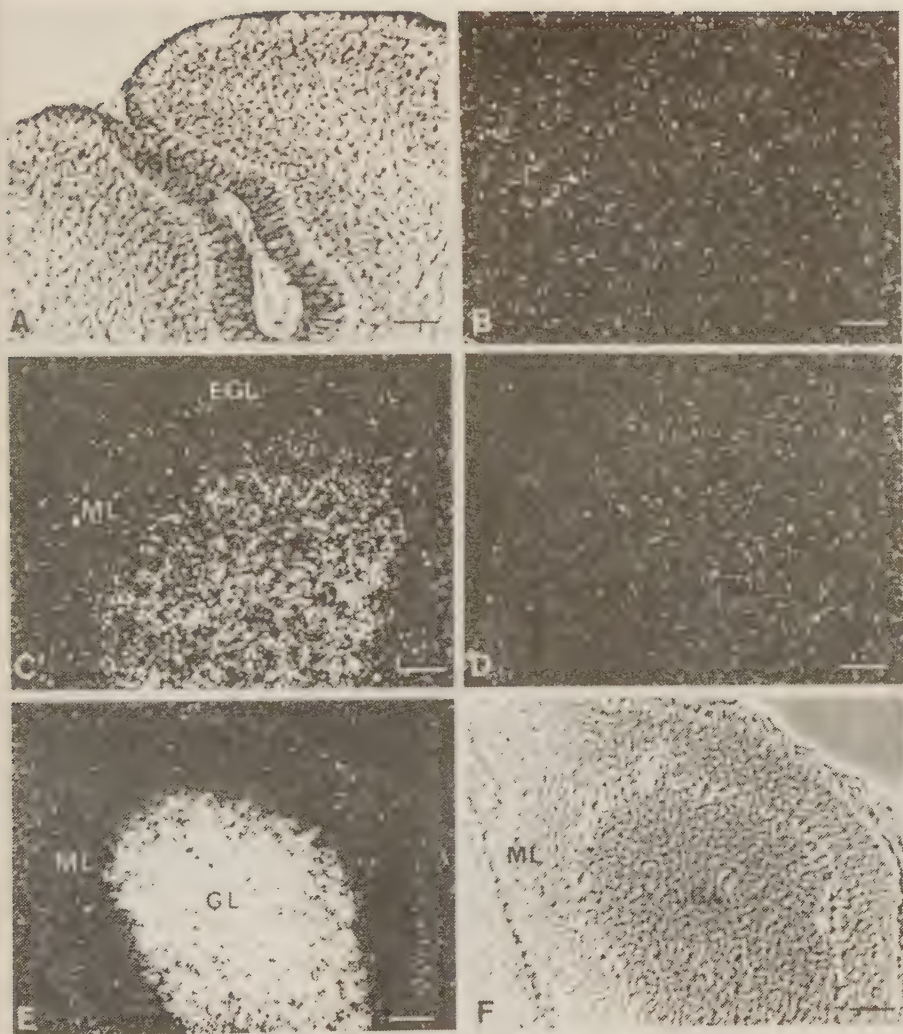


Fig. 12. Hybridization of ^{35}S -labelled synapsin cRNA probe to sagittal sections from the rat cerebellum.

(Panel A and F: phase contrast photomicrographs. Panel B-E: dark field photomicrographs, silver grains of the photoemulsion appear white.) Panel A and B: section from 5-day-old cerebellum; homogenous distribution of silver grains over the tissue reflecting only background hybridization. Panel C: 15-day-old cerebellum showing accumulation of grains over the GL but not the EGL. Panel D: hybridization with control synapsin cRNA (same sequence as mRNA) to 15-day-old cerebellum; background hybridization only. Panel E: 20-day-old cerebellum; high accumulation of silver grains over GL. Panel F: corresponding phase contrast picture to E. GL = granule cell layer, ML = molecular layer, EGL = external granule cell layer. Scale bar: 25 μm . Exposure time: 6 days.

3.2.4 Cellular localization

The potential for higher resolution offered by ^{35}S -labelled probes was exploited to define the neuronal cell type expressing synapsin mRNA. High magnification photomicrographs revealed heavy accumulation of silver grains over granule cells and only a small number of grains over Purkinje cells (fig. 13 D). Thus the expression of synapsin mRNA was demonstrated specifically in the population of granule cells undergoing a particular phase of differentiation, i.e. synaptogenesis.

In order to show that the variation in synapsin mRNA expression was not due to selective loss of poly(A)^+ RNA from particular cell types during the processing of the tissue, hybridization experiments were carried out using ^3H -poly(U). This method has been applied previously to quantify total poly(A)^+ mRNA in tissue sections. It was found that the abundance of autoradiographic grains from ^3H -poly(U)/ poly(A)^+ mRNA hybrids is directly proportional to the total poly(A)^+ RNA in cells (Hecht et al., 1981; Angerer et al., 1981). When this technique was applied to cerebellar slices of different ages poly(A)^+ RNA was detectable in high amounts in the various cell types of the cerebellum at all stages studied. (Data for two of these are shown in figure 13 A and C). This result further demonstrates the specificity of the synapsin mRNA hybridization.

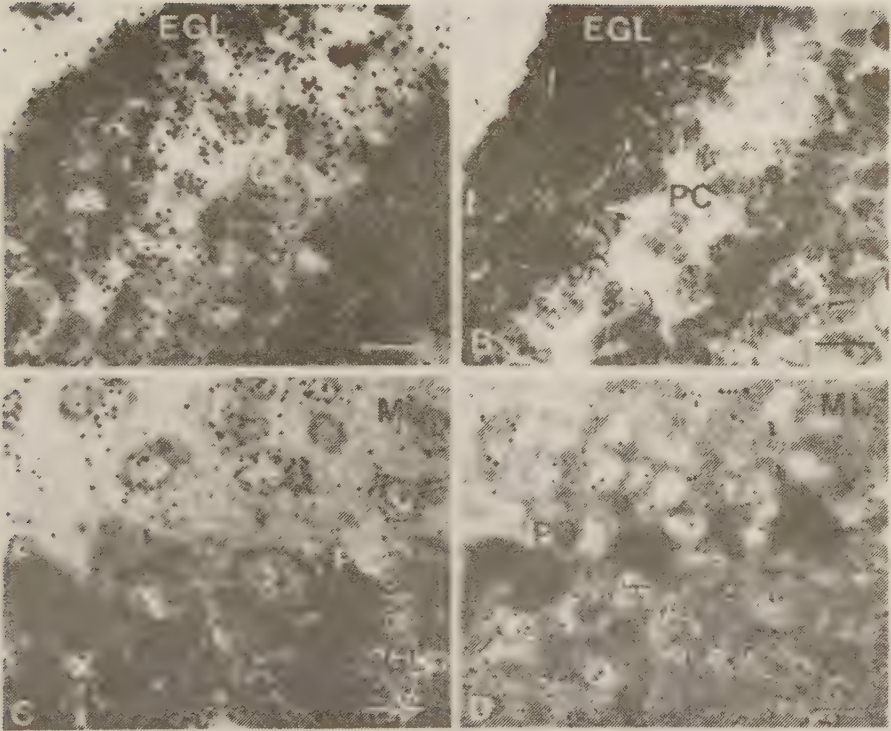


Fig. 13. Cellular localization of poly(A)⁺ RNA and synapsin mRNA in the rat cerebellum.

(Panel A and C: hybridization with ³H-poly(U), 9 day exposure. Panel B and D: hybridization with ³⁵S-synapsin-cRNA probe, 6 day exposure.) Panel A: section of 5-day-old cerebellum; accumulation of silver grains over neurons in EGL; PC and deeper layers of the immature cerebellum reflecting the poly(A)⁺ RNA content of the cells. Panel B: 5-day-old cerebellar section, no synapsin specific signal. Panel C: section of 20-day-old cerebellum, as in A high poly(A)⁺ content of interneurons in ML, PC and GL. Panel D: 20-day-old cerebellum; accumulation of silver grains over GL indicating synapsin specific signal, but not over PC (arrows). The dark colour of the PC results from incomplete destaining after thionin counter-staining. GL = granule cell layer; ML = molecular layer; PC = Purkinje cell layer. Scale bar: 5 μ m.

4. DISCUSSION

4.1 Two messenger RNA classes encode synapsin

Previous experiments had demonstrated the existence of two synapsin mRNA species in 10-day-old rat brain, 4.5 and 5.8 kb in length (Kilimann and DeGennaro, 1985). Using more accurate RNA standards (BRL RNA ladder) their sizes were newly determined to be 3.4 and 4.5 kb. It was proposed that each of these mRNAs might encode one of the two synapsin polypeptides Ia and Ib. However there are three lines of experimental evidence which suggest that each of these two mRNA species alone can give rise to both proteins. Firstly, polysomes purified from the brains of 10-day-old rats direct the translation of both polypeptides *in vitro* (DeGennaro et al., 1984) and have been shown to contain both the 3.4 and 4.5 kb synapsin mRNA (Kilimann and DeGennaro, 1985). Secondly, when mRNA is purified by immunoadsorption of these polysomes with synapsin-specific antibodies, both the 3.4 and 4.5 kb mRNAs are isolated. This could only occur if both synapsin transcripts were associated with polysomes bearing nascent synapsin polypeptides. *In vitro* translation reactions programmed by the immunoabsorbed mRNAs direct the synthesis of both synapsin polypeptides (Kilimann and DeGennaro, 1985).

The detailed Northern blot study described in Results was performed to assay the appearance of the two synapsin mRNAs during the development of the rat cerebellum, which was chosen as an *in vivo* model system. The results of the Northern blot study clearly demonstrated the differential regulation of the two synapsin mRNA species during the development of the rat cerebellum: the 4.5 kb transcript is detectable only from birth until postnatal day 7, whereas the 3.4 kb transcript is continuously expressed from birth into adulthood. A similar decrease in the 4.5 kb mRNA was also found in brain (minus cerebellum), implying that this pattern of regulation is a general phenomenon.

By contrast, the synapsin proteins Ia and Ib appear to be synthesized in a constant ratio at all stages of rat brain development, including adult (DeGennaro et al., 1983). The different developmental profiles of the two synapsin mRNAs and the two proteins now lead to the hypothesis that the 3.4 kb band alone can give rise to both proteins. In order to explain this result it is proposed that the 3.4 kb band consists of more than one mRNA species of similar length, which are not resolved by the agarose gel system used. Since the two synapsin polypeptides Ia and Ib differ only by 4-6 kD, i.e. approximately 40 amino acids, the putative mRNAs encoding them need only differ by 120 nucleotides. The existence of synapsin mRNA variants has been shown previously by DNA sequence analysis of synapsin specific cDNA molecules (McCaffery and DeGennaro, 1986). A similar heterogeneity within brain-specific mRNAs, detected by a brain-specific cDNA clone, was reported recently by Tsou et al. (1986). More pertinently, Ngai et al. (1987) have shown, that a single size class mRNA encoding multiple forms of chicken protein 4.1 contains several mRNAs of similar size.

The recent localization of the synapsin gene to the human and murine X chromosome (Yang-Feng et al., 1986) and Southern blot data of rat genomic DNA (Carroll and DeGennaro, unpublished observation) give strong evidence for the existence of a single copy of the synapsin gene in the haploid genome. The origin of the two transcripts observed and the microheterogeneity in mRNA suggested by the data presented must then be explained. The experiments described in section IV were designed to approach these questions. They will be there further discussed in detail.

4.2 Developmental regulation of synapsin gene expression

A quantitative Northern blot study was performed to investigate the developmental regulation of the 3.4 kb mRNA species in the rat cerebellum. The quantification of

the 3.4 kb mRNA species shows that a five-fold increase occurs between postnatal day 7 and 20. This time course suggests a strong correlation with the synaptogenesis of granule cell axons on Purkinje cell dendrites, the major event in cerebellar development at that time (Altman, 1972 a,b). A similar correlation has been found at the protein level, showing that the amount of synapsin increases to a maximum during the period of synaptogenesis in brain (Lohmann et al., 1978; DeGennaro et al., 1983). The peak level of the 3.4 kb mRNA species decreases to an adult level which is still quite high, indicating a constitutive synthesis of synapsin mRNA in neurons of adult animals. The correlation between high levels of synapsin gene expression and synaptogenesis is not surprising given that the synapsin protein is highly concentrated in virtually all nerve terminals of the peripheral and central nervous system (DeCamilli et al., 1983). The persistence of elevated levels of synapsin mRNA in the adult cerebellum may support the hypothesis that synaptic connections undergo constant remodelling (for a review see Cotman and Nieto-Sampedro, 1984). The process might involve the degradation of synapsin in old terminals and its *de novo* synthesis for newly-forming nerve endings.

4.3 Correlation of synapsin gene expression and granule cell synaptogenesis

The localization of synapsin mRNA during development by *in situ* hybridization provided additional insight into the temporal and spatial appearance of synapsin mRNA in relation to the state of differentiation of a particular neuronal cell type. As a model for a developing neuron the granule cell of the cerebellum was chosen. Synapsin mRNA could be localized from postnatal day 15 onward in the internal granule cell layer of the cerebellum. This coincides with the maximum level of synapsin mRNA demonstrated by quantitative Northern blot analysis. The

lower levels of expression of synapsin mRNA present in the cerebellum from birth until postnatal day 10 could not be visualized *in situ*, indicating the limit of detection. Using ^{35}S -labelled probes synapsin mRNA localization could be extended to the cellular level. Thereby it was possible to define the cell population responsible for the strong increase in synapsin mRNA expression during development as the granule cells of the internal granule cell layer. The accumulation of silver grains over the cell bodies of these neurons reflects synapsin mRNA production during the period of synaptogenesis of the parallel fibers on the Purkinje cell dendrites. In other cell populations of the cerebellum, such as the germinal matrix of external granule cells, the interneurons of the molecular layer or the Purkinje cells, synapsin mRNA levels were below the limit of detection. These findings are also in good agreement with the immunocytochemical localization of the synapsin protein in the molecular layer of the cerebellum during the same period of development (DeCamilli et al., 1983).

The specificity of the *in situ* hybridization procedure has been demonstrated by RNase A treatment of the tissue prior to hybridization; by the use of the non-hybridizing synapsin cRNA as a negative control and by demonstrating the poly(A)⁺ RNA content by hybridization with a ^3H -labelled poly(U)-probe. In summary the combination of quantitative Northern blot analysis and *in situ* hybridization histochemistry has proven to be a powerful means of evaluating neuron-specific gene expression at the cellular level during development.

4.4 Different levels of synapsin gene regulation

The result of Northern blot analysis and *in situ* hybridization have yielded the intriguing finding that the expression of the gene for synapsin, reflected by steady state levels of mature synapsin mRNA, is regulated in at least two ways.

Firstly, a developmentally coordinated decrease in the production of one of two synapsin mRNA classes which is probably the consequence of a change in differential processing of the primary transcript (see section IV). Another possible explanation could be that the disappearance of the larger mRNA species is due to changed RNA stability during development. Factors like the length of the poly(A) tail are thought to be directed correlated with RNA stability (Darnell, 1982) and could influence the stability of the synapsin mRNAs during development. Secondly, the overall level of expression of the synapsin gene is further regulated in a fashion coordinated with the major period of synaptogenesis.

Finally, a direct demonstration of the presence of high levels of synapsin mRNA in cells undergoing synaptogenesis has been demonstrated by *in situ* hybridization histochemistry. This opens up the possibility of a detailed mapping of the brain with a probe which identifies the cell bodies of neurons in the process of synaptogenesis.

The mechanism underlying regulation of the expression of synapsin mRNA at the time of synaptogenesis remains unknown. Further research is needed to distinguish between two possible interpretations of the data. Firstly, that the initiation of synapsin gene expression occurs in the innervating neuron as a result of some interaction between the innervating neuron and its target neuron. Secondly, that the initiation of synapsin mRNA synthesis does not require this cell-cell interaction, but rather is an event that is genetically programmed to occur at roughly the same time as the formation of synapses. The recent observation by Ellis et al. (1985) that synapsin protein is present in growth cones isolated from fetal rat brain support the latter interpretation by indicating that synapsin is already present in brain during axonal sprouting. A similar observation was reported by Mason (1986) who could localize synapsin-immunoreactivity in ingrowing afferent fibers of the embryonic mouse cerebellum.

IV. CHARACTERIZATION OF THE SYNAPSIN mRNAs AND THEIR STRUCTURAL RELATIONSHIP TO THE SYNAPSIN GENE

1. INTRODUCTION AND GOALS OF THE STUDY

1.1 How are the two synapsin mRNA species related to the synapsin gene?

As shown in section III of this study there are two mRNA species of 3.4 and 4.5 kb in size, which are detected by the synapsin 5E₂ cDNA fragment and whose expression is differentially regulated during neuronal development. Considering the finding that there is only one synapsin gene (Yang-Feng et al., 1986; Carroll and DeGennaro, unpublished observation) it was assumed that both mRNA species derive from this single gene. How do the two transcripts originate from this gene?

The production of multiple mRNAs from a single transcriptional unit is a well-characterized phenomenon in eucaryotic systems. It can involve alternative splicing of the primary transcript, the use of differential transcriptional start and/or polyadenylation sites or any combination of the above to produce multiple mature mRNAs (for a review see Leff et al., 1986). There are examples in which the protein-coding regions are unaffected by the splicing event and thus the consequences of alternative mRNA production remain obscure. In other cases, where the protein product is altered, alternative splicing of the primary transcript has an obvious result.

The selective regulation of RNA processing is of special importance in the control of developmental- or tissue-specific expression of certain genes. A prominent example is the IgM heavy chain gene. Alternative splicing allows the switch in expression of IgM heavy chain from a membrane associated to a secreted form during B-lymphocyte development (Alt et al., 1980; Rogers et al., 1980; Early et al., 1980). Tissue-specific expression regulated by selective RNA production was described for

the calcitonin/CGRP gene (Rosenfeld et al., 1981; Rosenfeld et al., 1982 ; Amara et al., 1982). Through alternative RNA splicing and changes in poly(A) site selection different RNAs are produced encoding the thyroid-specific calcitonin peptide and the calcitonin-gene-related-peptide (CGRP) which is predominantly found in nervous tissue.

In the case of the synapsin gene, selective RNA processing might be the means of controlling the developmentally regulated expression of the two transcripts during neuronal development. In order to elucidate this phenomenon it was necessary to obtain detailed information about structural differences between the two mRNAs and relate these differences to the structure of the synapsin gene. The synapsin gene covers at least 50 kilobases in the rat genome. Genomic clones have been isolated from a lambda genomic clone bank by hybridization with synapsin cDNA fragments. Exons representing the protein-coding region and the 3' untranslated region in the cDNA clone pSyn5 have been localized to specific genomic DNA fragments (Carroll, unpublished observation)(see also fig. 14).

However since it is not yet known which of the two mRNA species the pSyn5 cDNA clone is derived from, and since pSyn5 does not make up the full length of either of the two mRNAs, the exact limits of the synapsin gene transcription unit have not yet been defined. A more detailed knowledge of the structural relationship between the two synapsin mRNAs would focus attention on areas in the gene which potentially contain regions encoding the differences between the two mRNAs.

In order to provide further information concerning these questions several approaches were applied:

(i) Further characterization of the two mRNAs by hybridization with different synapsin cDNA fragments in order to study their degree of homology.

(ii) The RNase H assay was developed and applied to map differences with respect to their 3' or 5' extensions.

(iii) Hybridization with genomic fragments to Northern blots of brain RNA was performed to gain information about the exon/intron structure of the synapsin gene, and finally to elucidate the pathway of selective RNA production.

THE SYNAPSIN GENE



Fig. 14. Incomplete restriction map of the rat synapsin gene.

The gene covers roughly 50 kb in the rat genome. Exons (black boxes) have been mapped to different areas in the gene by hybridization with synapsin cDNA. (S = Sal I; E = Eco R1; B = Bam H1).

1.2 The RNase H assay

Ribonuclease H (RNase H) purified from *E. coli* (Berkower et al., 1973; Henry et al., 1973) or from calf thymus (Stafrianopoulos and Chargaff, 1973; Haberkern and Cantoni, 1973) is an endoribonuclease which specifically hydrolyses the phosphodiester bonds of RNA in an RNA/DNA hybrid molecule to produce 3'-OH and 5'-P products. It does not degrade single-stranded nucleic acids, duplex DNA or double-stranded RNA.

The special activity of RNase H has recently been used for the investigation of various problems such as: specific removal and length determination of poly(A) stretches of cellular mRNAs directed by hybridization with oligo(dT) (Sippel et al., 1974; Vournakis et al., 1975; Shaw et al., 1986); site-specific enzymatic cleavage of rRNA directed by hybridization with small oligonucleotides and subsequent sequencing of the cleaved products (Donis-Keller, 1979; Tapprich and Hill, 1986); and finally RNase H is commonly used in cDNA cloning procedures for the removal of the mRNA template after first-strand synthesis (Okayama and Berg, 1982; Gubler and Hoffmann, 1983).

In the present study the special properties of RNase H from *E. coli* were exploited to analyze the structural differences of the two synapsin mRNA species. Oligonucleotides deduced from the synapsin cDNA sequence (see Results; fig. 17) were used to direct specific cleavage of the mRNAs by RNase H. The resulting fragments were size-fractionated by agarose gel electrophoresis and blotted onto nitrocellulose. Through hybridization with 3' or 5' specific synapsin cDNA fragments the newly created mRNA fragments could be detected (fig. 15). Their sizes were determined by comparison with RNA molecular weight standards.

Using this approach length differences in the 3' or 5' extension of the two mRNAs could be analyzed and further conclusions were possible about the organization of the synapsin gene.

The RNase H assay

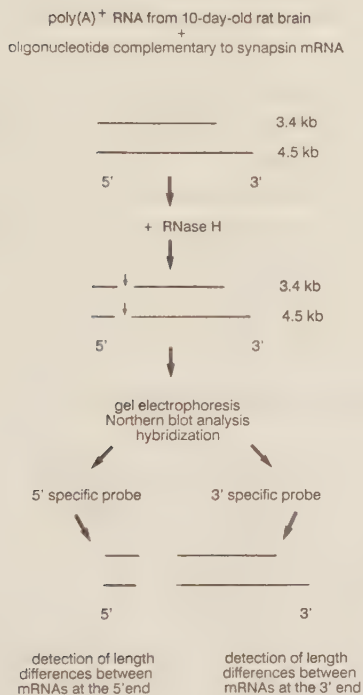


Fig. 15. The RNase H assay.

Oligonucleotide-directed cleavage of synapsin mRNAs by RNase H. Size-fractionation of the cleavage products by agarose gel electrophoresis and subsequent blotting onto nitrocellulose. Detection of 3' or 5' cleavage products by hybridization with radiolabelled synapsin cDNA fragments

2. MATERIAL AND METHODS

2.1 Synthesis of oligonucleotides

The sequences of two oligonucleotides (19mer and 23mer) were deduced from the synapsin cDNA sequence (for details see Results) and they were synthesized with an Applied Biosystem DNA synthesizer.

The yield of the synthesis was determined according to instructions of Fritz (Max-Planck-Institute for Biochemistry, personal communication). During DNA synthesis a trityl-group protects the 5' end of the growing DNA chain. It has to be removed prior to addition of a new nucleotide and thus can be used to determine the efficiency of each nucleotide addition step. The volume of each aliquot of the 18 or 21 samples, which reflect the single steps of the nucleotide addition cycles, was made up to 4 ml with acetonitrile. 100 μ l of this solution were added to 1 ml of 0.1 M toluol-4-sulfonic acid in acetonitrile; the absorption was measured at 498 nm for each sample. The average yield of each synthesis step was calculated using the following formula:

$$\lg x = \lg (\text{Fr } n) - \lg (\text{Fr } 1) / n - 1.$$

(X = percentage yield; Fr n = optical density of last fraction; Fr 1 = optical density of first fraction; n = number of fractions). The yield of each single synthesis step was typically 97 - 98%.

2.2 Purification of oligonucleotides by h.p.l.c.

After removal from the solid support of the DNA synthesizer the oligonucleotides were dissolved in 25% ammonia and heated to 55°C for 12 h to remove the blocking groups. Then the samples were lyophilized to dryness; resuspended in 1 ml H₂O each; lyophilized again and finally dissolved in 100 μ l of 0.1 M TEAA, (triethylammonium acetate), pH 7.0. The 5'-O-DMT - protected oligonucleotide was purified by chromatography

on a C-18 (250 mm x 4.6 mm) reverse phase column, flow rate 2 ml/min. The elution was isocratic for 2 min in 100% 0.1 M TEAA, pH 7.0, and then a gradient of 15 - 20% acetonitrile was applied over 39 min. The oligonucleotide (19mer or 23mer), detected by its absorbance at 260 nm, eluted after 25 min. It was collected and evaporated to dryness in a *Speed Vac* concentrator. The dry sample was taken up in 80% acetic acid and left at room temperature for 30 min to remove the trityl-group from the 5' end of the oligonucleotide. It was further processed by repeated lyophilization and resuspension cycles in H₂O and finally resuspended in a small volume and adjusted to a final concentration of 1 mg/ml after optical density reading at 260 nm. The yield of oligonucleotide synthesis after HPLC purification was typically 0.9 mg DNA.

2.3 RNase H digest of RNA/DNA oligomer hybrids

The protocol of Donis-Keller (1979) was modified and then applied for the RNase H digestion of poly(A)⁺ RNA from 10-day-old rat brain directed by the 19-base or 23-base oligonucleotide deduced from the synapsin cDNA. Each reaction mixture (10 µl) contained 2-4 µg of poly(A)⁺ RNA and 0.2 - 0.02 µg oligonucleotide in 50 mM Tris/HCl, pH 8.0, 4 mM MgCl₂, 1 mM DTT, 0.03 mg/ml BSA (RNase free, Pharmacia). The reaction mixtures were heated to 50°C for 10 min and subsequently cooled to 37°C. Then 0.03 units of RNase H (*E. coli* SK 780, Pharmacia) were added and the reaction allowed to proceed at 37°C for 30 min. The digest was stopped by addition of 2 µl 0.2 M EDTA. The reaction volume was made up to 20 µl with sterile H₂O; 2 µl of 3 M sodium acetate and 65 µl of 95% ethanol were added; mixed thoroughly and the RNA was precipitated by cooling in a dry-ice ethanol bath for 15 min. The RNA was recovered by centrifugation at 12000 rpm and 4°C. The RNA pellets were dried briefly under vacuum, resuspended in glyoxylation mixture for denaturation and further analysis by agarose gel electrophoresis as described

earlier. For control purpose, mRNA samples in water or buffer only, plus or minus RNase H were carried through the procedure exactly as described above.

2.4 Labelling of genomic fragments

The modified technique of radiolabelling DNA restriction fragments developed by Feinberg and Vogelstein (1984) was applied to label genomic fragments of the synapsin gene to high specific activity. This procedure offers the advantage of directly labelling DNA restriction fragments in low-melting agarose omitting the purification step from the gel which is usually necessary for nick-translation. Through the use of random oligonucleotides as primers and the *Klenow* fragment of *E. coli* polymerase I the DNA is labelled to high specific activity in the presence of ^{32}P -labelled deoxynucleoside triphosphates.

Synapsin gene fragments cloned into the phage vector *EMBL 3* were cleaved with *Eco* RI or *Bam* HI restriction enzymes and the resulting fragments were electrophoretically separated on a 0.8% low melting point agarose gel (BRL) in TEA buffer. After electrophoresis the gel was stained with ethidium bromide (1 $\mu\text{l/ml}$) for 30 min. The desired restriction fragment was visualized under UV-light, excised cleanly and put into a preweighed 1.5 ml eppendorf tube. H_2O was added at a ratio of 3 ml $\text{H}_2\text{O/g}$ of gel. The tube was then placed in a boiling water bath for several minutes to dissolve the agarose and denature the DNA. Samples prepared in this way could be stored at -20°C or used immediately for the labelling reaction.

The labelling procedure was performed as follows: The DNA fragment of interest (10 - 50 ng) in the melted agarose solution was made up to 32.5 μl with H_2O , then 2 μl of DNase-free BSA (10 mg/ml; Pharmacia), 10 μl of oligo-labelling buffer (- dCTP)*, 5 μl of ^{32}P -dCTP (NEN, 3000 Ci/mmol) and 4 units of *Klenow* fragment of *E. coli* polymerase I (Pharmacia) were added to give a final volume of 50 μl . The labelling reaction was carried out

overnight at room temperature and stopped by the addition of 200 μ l stop solution containing 20 mM NaCl, 20 mM Tris/HCl, pH 7.5, 2 mM EDTA, 0.25% SDS.

Incorporation was measured by the DE-81 filter assay. Usually the amount of input radionucleotide incorporated reached 60 - 80% and the resulting labelled DNA had a specific activity of ca. 10^9 cpm/ μ g. These values were in excellent agreement with those reported by Feinberg and Vogelstein (1984). The labelled DNA fragments could be directly used for Northern or Southern blot hybridization without further purification.

*(OLB = Oligo-Labeling Buffer was made as follows:

Solution O: 1.25 M Tris-HCl, 0.125 M $MgCl_2$, at pH 8.0.

Solution A: 1 ml solution O + 18 μ l 2-mercaptoethanol + 5 μ l dATP, 5 μ l dTTP, 5 μ l dGTP (0.1 M each).

Solution B: 2 M Hepes, titrated to pH 6.6 with 4 M NaOH. Solution C: Hexadeoxyribonucleotides (Pharmacia) evenly suspended in TE at 90 OD units/ml. To make the OLB buffer the solution A,B,C, were mixed in ratio of 100:250:150.)

2.5 Probes and condition for hybridization

The probes for the Northern blot experiments of section IV were two Eco RI restriction fragments of the synapsin cDNA clone pSyn5 and a short synapsin cDNA clone pSyn13. The conditions for Northern blot hybridization were exactly as described. Characteristics of the probes (see also fig. 16):

Name	recognition area	length

E ₃	5' end	400 bp + 800 bp vector
E ₂	core sequence	1700 bp
pSyn13	3' end	600 bp + vector

3. RESULTS

3.1 Hybridization with different synapsin cDNA fragments

Results of experiments described in section III show that both synapsin mRNA species were present in RNA from 10-day-old rat brain (detected by hybridization with the E₂-probe which represents the core region of the synapsin cDNA pSyn5). In order to investigate further homologies between the two synapsin mRNA species 5' and 3' specific cDNA fragments derived from pSyn5 and pSyn13 were used for hybridization (fig. 16).

Poly(A)⁺ mRNA from 10-day-old rat brain was size-fractionated on a 1.2% agarose gel, blotted onto nitro-cellulose and hybridized with ³²P-labelled synapsin cDNA fragments derived from different parts of the cDNA. As shown in figure 16 hybridization with E₃ (specific for 5' end of the cDNA), E₂ (core part of the cDNA) and pSyn13 (specific for 3' end of the cDNA) did not detect any significant differences in the pattern of the two synapsin mRNA species. Both appeared to hybridize with the three different synapsin probes with similar ratio of intensity. The 3.4 kb transcript gave a much stronger signal than the larger 4.5 kb transcript in all three cases. The slight variations in overall signal intensity between A; B and C in figure 16 were caused by different specific activities of the probe preparations.

The conclusion of this experiment is that both synapsin mRNAs share a high degree of homology with the whole synapsin cDNA sequence. In order to define the precise difference between the two transcripts which appear to be homologous over about 3200 nucleotides, but still differ by about 1000 bases in length, the RNase H assay was applied.

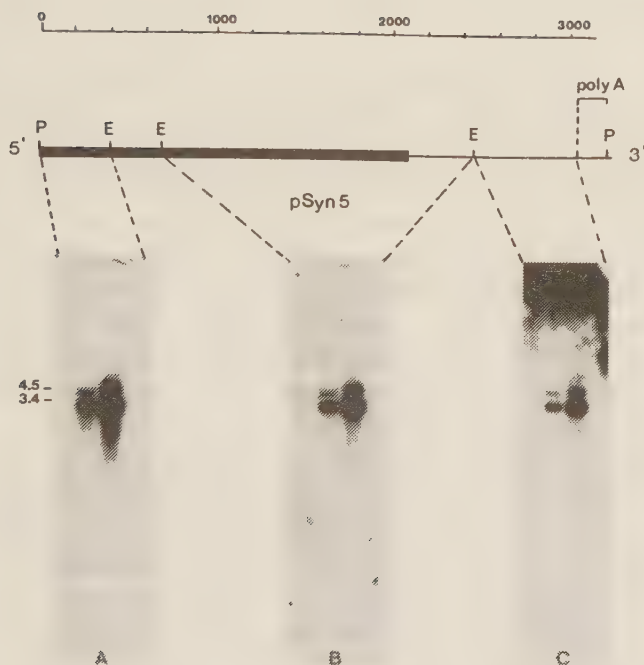


Fig. 16. Homology between the two synapsin mRNA species.

Panels A, B and C show autoradiograms of RNA blot hybridizations between total poly(A)⁺ RNA of 10-day rat brain and nick-translated probes complementary to three regions of the synapsin-specific cDNA pSyn5. Panel A: synapsin cDNA fragment E₃. Panel B: synapsin cDNA fragment E₂. Panel B: synapsin cDNA clone pSyn13. Scale at the top is in base pairs. Protein-coding region in pSyn5 is indicated by the bold line. RNA blots and hybridizations were carried out as described in Methods. Each filter strip shows two lanes containing 2 and 4 μ g of rat poly(A)⁺ RNA, respectively.

3.2 Important parameters for the RNase H assay

During the development of the oligonucleotide-directed RNase H assay several critical parameters became apparent:

3.2.1 Nonspecific degradation by contaminating RNases

Above all it was essential to avoid any contamination of the reactions with RNases. Nonspecific degradation drastically reduced the number of RNA molecules available for the specific oligonucleotide-directed digestion by RNase H. Therefore all working material had to be sterile or RNase-free (autoclaved eppendorf tubes, pipette tips etc.) The digestion buffer had to be prepared from RNase-free stock solutions; only RNase-free BSA (Pharmacia) could be used for the RNase H buffer, since ordinary BSA (Miles) turned out to be the main source of RNase contamination in the reactions.

The annealing period for the formation of the mRNA/oligomer hybrids had to be as short as possible: I started with an annealing period of 1 h according to Donis-Keller, (1979), but found a short incubation of 5 min at 37°C to be sufficient prior to addition of RNase H. This drastically reduced nonspecific degradation of the RNA in the reactions.

3.2.2 Effect of oligonucleotide concentration

The concentration of oligonucleotides in the reaction proved to be critical for the specificity of the RNase H digest. A prerequisite for the successful digest was a great molar excess of oligonucleotide over RNA molecules. The optimal ratio was 0.1 - 0.01 μg oligo/ μg RNA (a 2,000 - 20,000 times molar excess of oligonucleotide over synapsin mRNA molecules) for the oligonucleotides to find their specific synapsin target mRNAs in the heterogenous

poly(A)⁺ RNA population.

Alterations in the oligo/RNA ratio had the following effects:

If the oligonucleotide concentration dropped below a critical concentration, the synapsin mRNA population was only partially digested. Concentrations of oligomers in excess of the optimal ratio led to nonspecific binding at "mismatch" sites within the synapsin mRNA sequence. This phenomenon resulted in a greater number of cleavage products than expected and disturbed the clarity of the result (Fig. 20). The "mismatch" sites, however, could clearly be predicted by comparison of the sequence of the oligonucleotide with the cDNA sequence (see also fig. 20).

3.3 Mapping of the two synapsin mRNA species by the RNase H assay

3.3.1 Results of cleavage directed by oligo II

As described earlier the two synapsin mRNAs appear to originate from a single synapsin gene (Yang-Feng et al., 1986). The result of the experiment described in section 3.2 shows that the two mRNAs share a high degree of homology with the entire 3.2kb of the synapsin cDNA sequence. What then accounts for the difference of 1 kb in the length of the two mRNAs? The RNase H assay was applied in order to study how the synapsin mRNAs differ with respect to their 3' or 5' ends.

Oligonucleotide II, a 19mer, complementary to the synapsin cDNA at a site 480 bases downstream of the 5' end of pSyn5, was used to direct digestion of the two synapsin mRNAs by RNase H (fig. 17). The binding site lies within the protein-coding region of the synapsin cDNA. Therefore it was assumed that oligo II would find its complementary binding site in both mRNA species.

After size-fractionation and blotting, 3' cleavage products were visualized by hybridization with the E₂-probe. Figure 18 B (lane 2 and 3) shows that the E₂-probe detected two cleavage products of 2.6 and 3.7 kb in size. This result indicated that the difference in length of about 1 kb between the two mRNAs was still maintained after the oligonucleotide-directed cleavage close to the 5' end. Therefore it was concluded that the size difference between the two synapsin mRNA species lies at their 3' ends.

To further substantiate this result the same digest was performed followed by hybridization with the E₃-probe which is complementary to sequences 5' to the oligo-directed RNase H cleavage site. This probe detected a single 0.8 kb fragment reflecting the extension of both synapsin mRNAs 5' to the oligonucleotide cutting position (fig. 18 A, lane 2 and 3).

The specificity of the digest was proven by control

reactions containing only RNA and RNase H. In these samples only the original-sized synapsin mRNAs were detectable proving that RNase H does not cleave in the absence of oligonucleotide (fig. 18 A,B; lane 1).

The precise length determination of the cleavage products was performed by comparing the migration distance of the individual products with a standard curve, plotted from the migration of ethidium bromide stained marker RNAs (BRL RNA Ladder) (fig. 19).

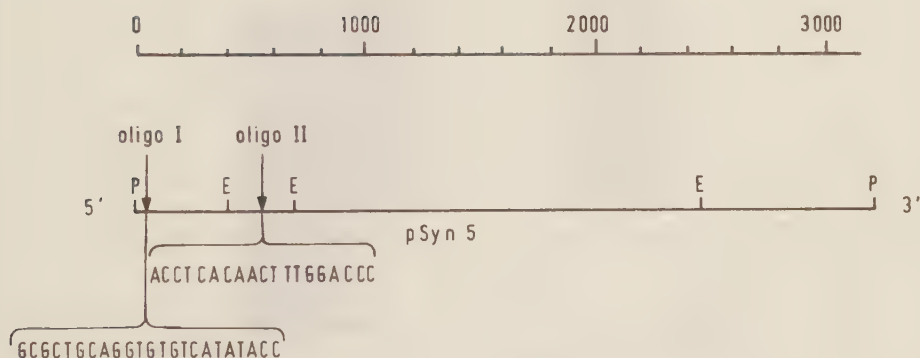


Fig. 17. Restriction map of synapsin cDNA pSyn5 and location of oligonucleotides I and II (deduced from pSyn5) within the cDNA sequence.

Sequences of oligonucleotides, which are complementary to the synapsin mRNA, are indicated in parentheses. (P = Pst I; E = Eco R1).

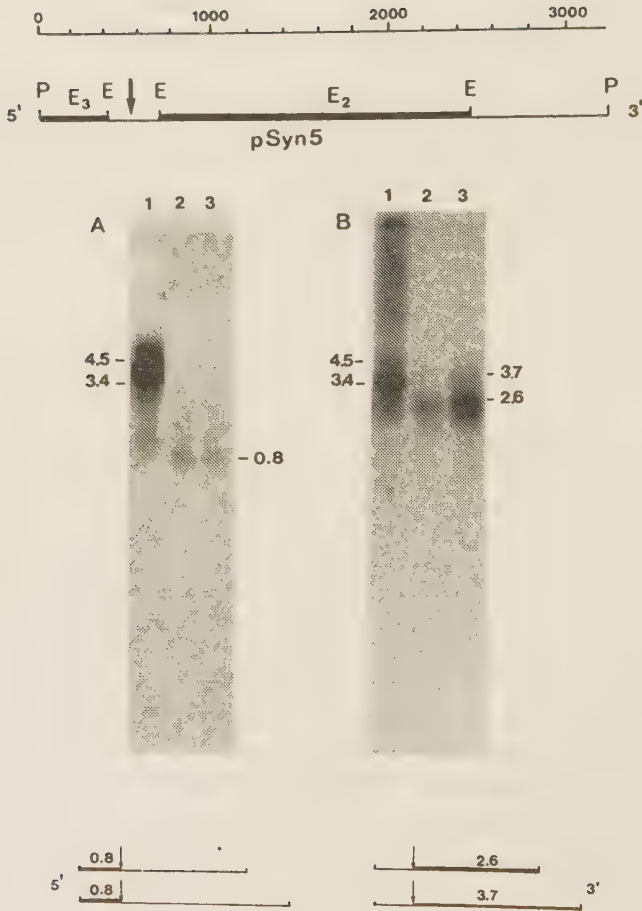


Fig. 18. Oligonucleotide-directed cleavage of synapsin mRNA by RNase H.

After oligonucleotide II-directed cleavage of rat brain poly(A)⁺ RNA by RNase H, as described in Methods, the resulting RNA fragments were displayed by agarose gel electrophoresis, blotted to nitrocellulose and hybridized with ³²P-nick-translated probes derived from synapsin cDNA clone pSyn5. Scale at the top is in base pairs. Bold lines in the pSyn5 restriction map indicate position of fragments E₃ and E₂ used as probes. Arrow indicates site of oligonucleotide sequence in clone pSyn5.

Panel A: Hybridization with 5' probe E₃. Lane 1: RNA + RNase H. Lane 2 + 3: RNA + oligo + RNase H. **Panel B:** Hybridization with 3' probe E₂. Lane 1: RNA + RNase H. Lane 2 + 3: RNA + oligo + RNase H. Drawings below Panel A and B show schematically the results of the cleavage.

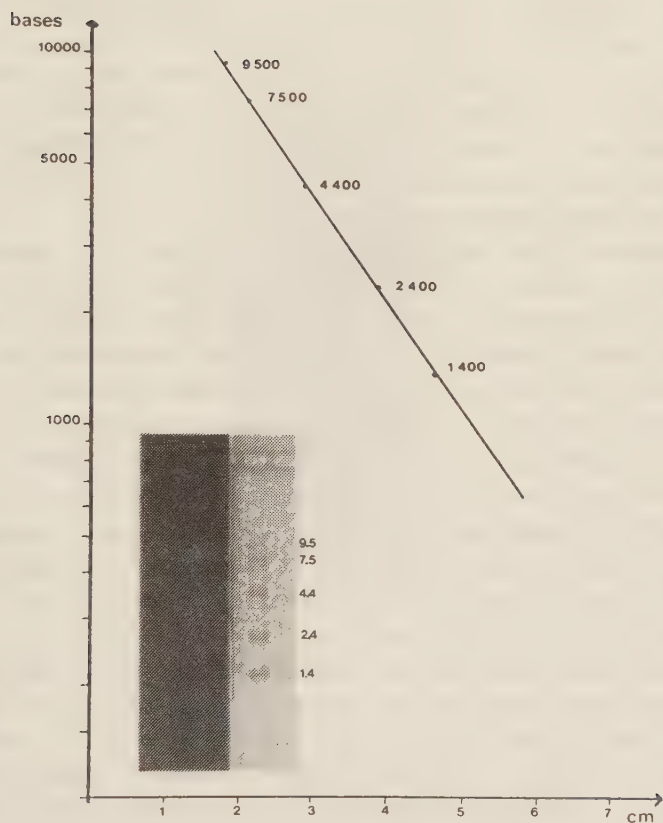


Fig. 19. BRL RNA Ladder standard curve for size estimation of RNase H cleavage product.

3 μ g RNA molecular weight standards were glyoxylated and size-fractionated on a 1% agarose gel adjacent to RNase H cleavage products. After electrophoresis the marker lane was cut off, stained with ethidium bromide and photographed next to a ruler. The migration distance of the individual RNA Ladder components was plotted versus their molecular weight. Note the remarkable linearity within a size range of 1.4 - 9.5 kb.

3.3.2 Results of cleavage directed by oligo I

In the preceding section the difference between the two synapsin mRNAs was detected after oligo II-directed cleavage, using 5' and 3' specific cDNA probes. For further confirmation of these results the advantage and flexibility of the oligonucleotide directed RNase H assay was exploited by using an oligonucleotide deduced from a different part of the synapsin cDNA. Oligo I, a 23mer, is complementary to a sequence 30 bases downstream of the 5' end of the cDNA pSyn5 (see fig. 17).

Based on the results described above for cleavage directed by oligo II, fragments of 4.2 and 3.1 kb were predicted (detected by 3' probe E₂), when oligo I was used to direct cleavage. Figure 20 shows the result of an experiment using oligo I: the sizes of the 4.5 and 3.4 kb mRNAs were in fact reduced to 4.2 and 3.1 kb (fig. 20, lane 1). Although the intensity of the two bands was weak, they confirmed the result that the two mRNAs differ at their 3' ends by 1000 bases. The smaller cleavage products also visible (1.5 and 1.7 kb), resulted from hybridization of oligo I at mismatch sites at position 224 and 1724 within the cDNA .

The specificity of the digest was proven by control reactions containing RNA and RNase H (fig. 20; lane 3;5), or RNA plus buffer alone (fig. 20; lane 4,6). In these samples only the original sized synapsin mRNAs were detected.

Through the use of two different oligonucleotides (Oligo I and II) to direct the cleavage of the two synapsin mRNA species by RNase H it was observed that the 1000 base difference apparent in their sizes occurs in the 3' portion of the molecules. Moreover it was shown that both mRNAs have the same extension 5' from the binding site of oligo II. This finding strongly suggested the use of a single transcriptional start site for the synthesis of both mRNA species.

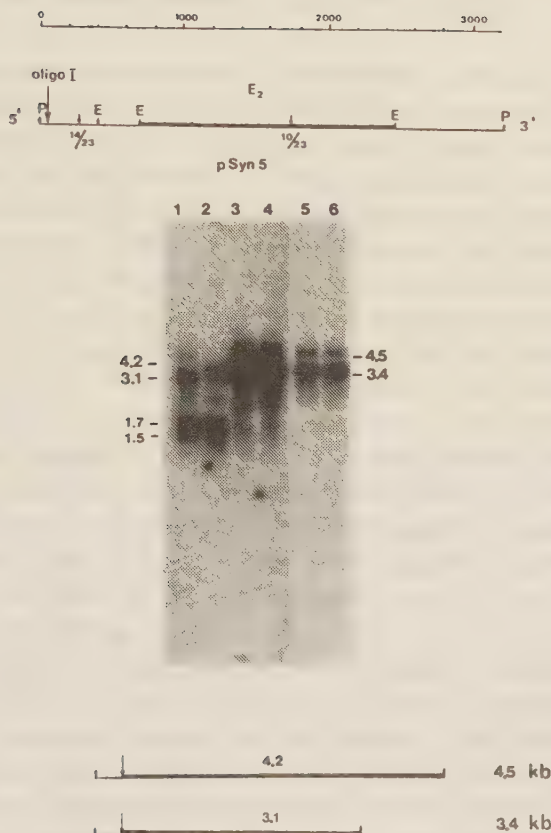


Fig. 20. Oligo I-directed cleavage of synapsin mRNAs by RNase H.

Diagram at the top shows the restriction map of synapsin cDNA pSyn5. Arrow indicates position of oligo I. Bold line indicates probe E_2 used for visualizing the RNase H cleavage products. Small arrows show position of mismatch sites within the cDNA sequence; numbers below these arrows indicate the degree of complementarity of oligo I at these sites. The autoradiogram below shows a Northern blot of oligo I-directed RNase H cleavage of poly(A)⁺ RNA from 10-day-old rat brain hybridized with radiolabelled synapsin cDNA fragment E_2 as described in Methods. Lane 1 and 2: RNA + RNase H + oligo I. In lane 1 successful cleavage (4.2 and 3.1 kb) plus "mismatch" cleavage (1.5 and 1.7 kb). In lane 2 only mismatch cleavage (1.5 and 1.7 kb) and residual uncleaved material. Lane 3 - 6: control reactions. Lane 3 + 5: RNA + RNase H; they differ only in the amount of RNA loaded. Lane 4 + 6: RNA + buffer.

3.4 Analysis of the structural differences between the two synapsin mRNAs on the genomic level

Hybridization with different synapsin cDNA fragments and the RNase H assay clearly demonstrated that both synapsin mRNA species share a high degree of homology and that the sequence difference of 1000 bases mapped to their 3' portions. The data available so far still leave open two possibilities for the generation of this size difference: a simple addition of 1000 bases at the 3' end by the use of different polyadenylation sites or the use of an alternatively spliced exon which gives rise to the extra 1000 bases of the 4.5 kb mRNA.

In order to answer this question the analysis was extended to the genomic level. In the diagram of figure 21 a partial restriction map of the synapsin gene is shown (Carroll, unpublished observation). By hybridization with synapsin cDNA fragments, exons have been mapped to particular restriction fragments covering a region of 50 kb in the rat genome. The most 3' exon-containing gene fragment is a 600 bp *Eco* RI fragment.

Attention of this study was focused on the 3' end of the synapsin gene, where the difference between the two mRNA species was assumed to be encoded. DNA restriction fragments of this area, prepared from cloned genomic DNA, were labelled by the Feinberg and Vogelstein technique (1984) and used as hybridization probes against Northern blots containing poly(A)⁺ RNA from 10-day-old rat brain. The approach was designed to identify sequences unique to the 4.5 kb mRNA and further to define the 3' border of the synapsin gene.

In initial experiments, where large DNA restriction fragments (1.7 kb *Bam* HI/*Eco* RI, 1.25 kb *Eco* RI, 1.05 kb *Eco* RI/*Sal* I, 3 kb *Eco* RI/*Bam* HI, 4 kb *Bam* HI) were used for the analysis, the following results were obtained (see fig. 21):

(i) No DNA fragment within 10 kb of the gene 3' to the 600 bp *Eco* RI fragment hybridized to either of the two mRNA species (fig. 21 B, lane 2 and 3). This result indicated the 3' border of the synapsin gene.

(ii) The 1.7 kb *Bam* HI/*Eco* RI fragment detected both mRNA species as expected, because it contains an exon mapped by hybridization with the cDNA fragment E_2 (fig. 21 B; lane 1). It recognized, however, the 4.5 kb mRNA species with a stronger relative intensity than the cDNA fragment E_2 (fig. 21 A, lane 1,2). This finding strongly suggested that sequences specific to the 4.5 kb mRNA were contained within this 1.7 kb fragment of the synapsin gene. Subsequently attention was focused on this area of the synapsin gene.

From previous experiments it was known that an E_2 -specific exon was located close to the 5' border of the 1.7 kb genomic fragment (Carroll, unpublished observation)(see also fig. 21). Therefore *Pvu* II fragments, located downstream of this exon, were chosen for the detailed analysis of this area (fig. 22). Northern blot analysis with these radiolabelled fragments as probes against poly(A)⁺ RNA clearly identified a 740 bp *Pvu* II fragment which contained sequences preferentially hybridizing with the 4.5 kb mRNA species (fig. 22; lane 2). A 940 bp fragment located more 3', however, clearly recognized both mRNAs with a pattern similar to that seen for E_2 (fig. 22; lane 3). These results clearly indicate that the 740 bp fragment carries sequence information unique for the 4.5 kb mRNA. The implications of these results are:

(i) Both synapsin mRNAs seem to have a common 3' end in the 600 bp *Eco* RI fragment, indicating the 3' border of the synapsin gene. The final proof has to be given by sequence analysis.

(ii) The size difference between the two synapsin mRNAs is probably due to the insertion of an alternative exon, located within the 740 bp *Pvu* II fragment, which generates the larger mRNA species.

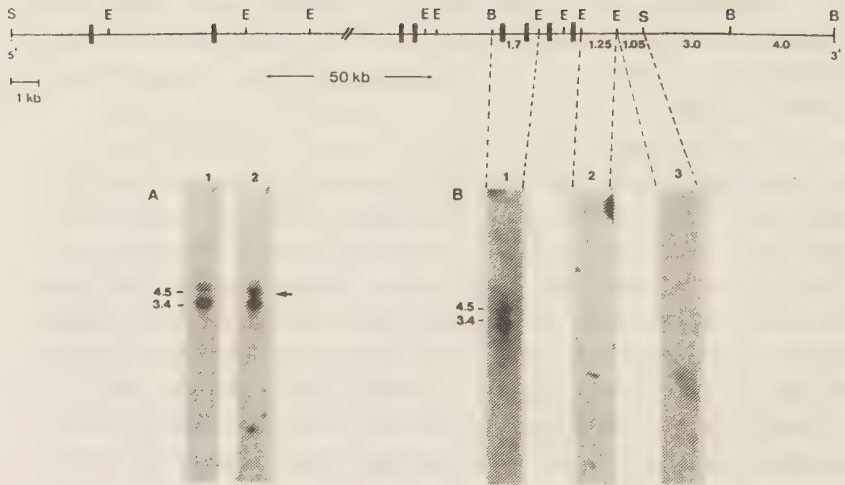


Fig. 21. Northern blots of poly(A)⁺ RNA from 10-day-old rat brain hybridized with radiolabelled genomic fragments as described in Methods.

Upper panel shows a restriction map of the synapsin gene. Black boxes signify exons (not in scale). Numbers below the map indicate the sizes of restriction fragments (in kb), which were used for hybridization. **Panel A**, lane 1: ³²P-nick-translated cDNA fragment E₂. Lane 2: genomic 1.7 kb B/E fragment, arrow points out stronger intensity of 4.5 kb mRNA. **Panel B**, lane 1: 1.7 kb B/E fragment; lane 2: 1.25 kb Eco R1 fragment; lane 3: 1.05 kb E/S fragment. (E = Eco R1; S = Sal I; B = Bam HI). Northern blots with 3.0 and 4.0 kb fragment as probes not shown.

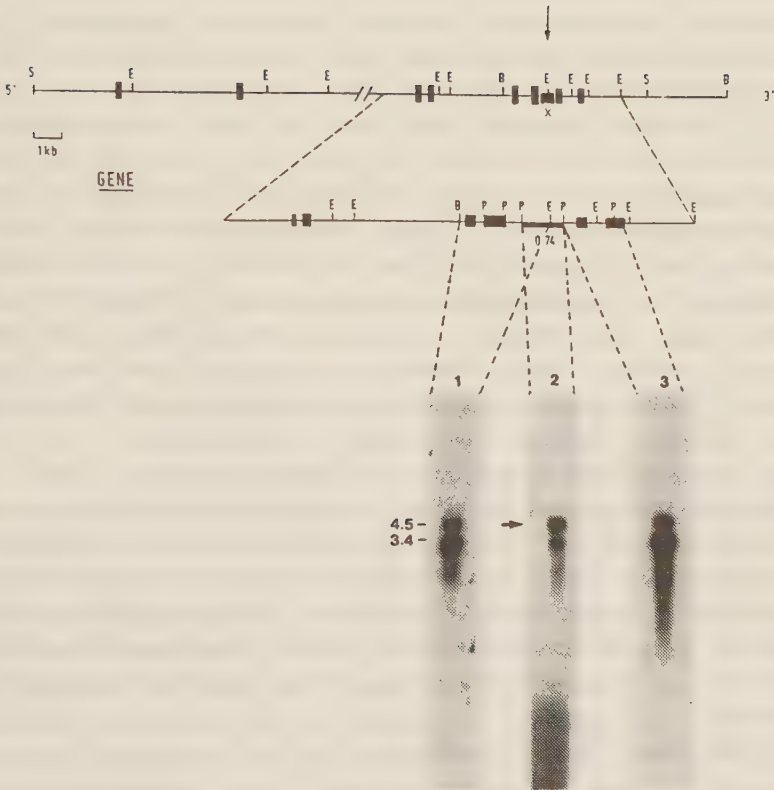


Fig. 22. Identification of an alternatively spliced exon in the synapsin gene.

Upper panel: restriction map of the synapsin gene as in fig. 21 with amplified map including Pvu II restriction sites. Black boxes indicate exons. Position of alternative exon (X) emphasized by arrow. Dotted lines indicate fragments used for Northern blot hybridization below. **Lane 1 - 3:** Northern blots of poly(A)⁺ RNA from 10-day-old brain, hybridized with radiolabelled genomic fragments as described in Methods. **Lane 1:** 1.7 kb B/E fragment; **lane 2:** 0.74 kb Pvu II fragment; **lane 3:** 0.94 kb P/E fragment. (E = Eco R1; P = Pvu II; B = Bam H1)

4. DISCUSSION

4.1 Methodological considerations concerning the RNase H approach

4.1.1 Advantages of oligonucleotide-directed cleavage

Oligonucleotides offer several advantages compared with single-stranded DNA fragments to direct specific cleavage of RNA molecules by RNase H:

If cDNA sequence data are available, complementary to the mRNA molecule(s) of interest, a set of oligonucleotides (i.e. oligo I and II) can easily be synthesized to direct cleavage at different sites in the RNA molecule. By contrast, use of several single-stranded cDNA probes requires subcloning each cDNA fragment into M 13 and the preparation of single-stranded phage pools for each experiment. Oligonucleotides, however, are available easily in large amounts and the yield of one synthesis is sufficient to perform many RNase H experiments.

The annealing reaction of mRNA/DNA hybrids prior to RNase H digestion is less time consuming with short oligomers (5 min - 1 h) according to my results and Donis-Keller (1979), in contrast to ssDNA fragments which require 3 - 12 h for annealing (Chien-Tsung Hu and Davies, 1986; Favaloro et al., 1980). The short annealing period of oligomer/RNA hybrids, which must be followed by RNase H digestion and agarose gel electrophoresis, permits the whole experiment to be performed within several hours. Taken together, the oligonucleotide-directed approach is quick, inexpensive, flexible and reliable.

4.1.2 Specificity of the RNase H assay

The specificity of the oligonucleotide-directed digest was proven by control reactions performed in the absence of oligomer. In these samples no digestion products were observed, indicating the specificity of the oligo-directed cleavage by RNase H.

As described in Results, oligonucleotide directed cleavage by RNase H sometimes occurred at "mismatch-sites" within the mRNA molecules. The use of these partially homologous sites was dependent on the oligo/RNA ratio in the reaction: too high an excess of oligomers (1 μg oligo/ μg RNA) resulted in nonspecific binding to partially complementary sequences; a decrease of the oligonucleotide concentration eliminated this effect. The position of mismatch sites in the RNA molecule was predictable by computer search of the cDNA sequence for regions of partial homology. In experiments performed with oligo I the partial complementarity was 14 out of 23 bases (position 224 in the cDNA) and 10 out of 23 bases (position 1724) within the mRNA molecules. RNA fragments (1.5 and 1.7 kb), observed in oligo I-directed cleavage experiments (fig. 20; lane 1 and 2) were in agreement with the use of these predicted mismatch sites and therefore did not seriously disturb the clarity of the result.

The detection of RNA fragments produced by cleavage at "mismatch" sites suggests that the RNase H digestion conditions (37°C, no salt), though quite stringent, still allow some mismatch hybridization by the oligonucleotides. However, more stringent conditions (i.e. higher temperature of incubation) were not compatible with optimal activity of the enzyme.

4.1.3 Effect of secondary structure on RNase H activity

Secondary structure did not appear to influence the activity of RNase H. All predicted oligo/RNA hybrids were cleaved (including those produced at mismatched sites) and therefore their accessibility was not masked by secondary structure. This assumption was supported by the observation of Donis-Keller (1979) that the activity of *E. coli* RNase H is less sensitive to effects of secondary structure when compared with the activity of calf thymus RNase H. Moreover, Donis-Keller rigorously tested the ability of *E. coli* RNase H to cleave at oligo/RNA hybrid

sites within a large RNA molecule by successfully cutting at different sites within the 1.240 kb STNV RNA (satellite tobacco necrosis virus) with this enzyme. In the case of oligonucleotide-directed cleavage of the synapsin mRNAs the cutting sites were located 800 or 300 bases distal to the 5' end of the mRNAs and were obviously accessible for the RNase H attack.

4.1.4 Resolution of the agarose gel system

The 1.2% agarose gel system used for the size fractionation of the cleaved RNA products has limited resolution. Although defined RNA standards (BRL RNA ladder) in a size range of 9.5 kb - 1.3 kb were used to determine the size of the cleaved products, the measured length may vary by 100 - 200 bases from experiment to experiment reflecting the limit of resolution of the agarose gel system.

4.1.5 Sensitivity

The autoradiographic signal of the 5' 0.8 kb fragment resulting from oligo II-directed cleavage and detected by the E₃-probe, appeared to be very faint in comparison with the corresponding 3' fragments. There are several possible reasons for this phenomenon:

The retention capacity of RNA molecules by nitrocellulose decreases with RNA size (Thomas, 1980). Therefore the 0.8 kb fragment may be not as well retained on the blot as the corresponding larger 3' RNA fragments. Since the 400 bp E₃ synapsin cDNA fragment used as probe in this experiment was part of a larger DNA fragment (1200 bp) containing pBR322 sequences the weaker signal intensity may derive from presence of less synapsin specific sequences in the hybridization reaction. Both possibilities discussed above probably contribute to the weak hybridization signal observed for the 5' specific 800 base fragment.

4.2 Structure of the synapsin mRNAs and the RNA processing pathway through which they are generated

The aim of the experiments outlined above was to investigate the degree of homology between the two synapsin mRNA species and to learn more about their structure. This analysis was extended to the genomic level in order to provide more information about the organization of the synapsin gene and finally to elucidate the processing pathway through which the mature synapsin mRNAs originate.

4.2.1 The two synapsin mRNAs species show a high degree of homology

Hybridization of different synapsin cDNA fragments to poly(A)⁺ RNA from 10-day-old rat brain showed that both mRNA species share a high degree of homology over the 3.2 kb sequence of the synapsin cDNA. Both the 3.4 kb and the 4.5 kb mRNAs hybridized to the 5' (E₃), core (E₂) and 3' (psyn13) cDNA fragments with a constant ratio of intensity. This type of analysis, however, cannot rule out the existence of partial sequence differences within the 3.2 kb sequence examined. For instance, if the larger 4.5 kb mRNA contained deletions or insertions within the large core region complementary to E₂, these differences probably would not be detected by hybridization with E₂. The smaller 5' and 3' flanking cDNA fragments were more likely to reveal sequence differences due to their small size (400 bp).

On the genomic level, the high homology between the two mRNAs argues for the existence of exons common to both mRNAs. In fact such exons have been demonstrated by hybridization of synapsin cDNA fragments (complementary to both mRNAs) to restriction fragments of synapsin specific genomic clones (Carroll, unpublished observation). However, alternatively spliced exons encoding sequences unique to the larger mRNA cannot be detected

with this type of analysis, because the cDNA detects only sequences common to both mRNA species.

4.2.2 The synapsin mRNAs have common 5' but diverging 3' extensions

The RNase H assay was applied for a more refined analysis of the origin of size differences between the two mRNAs in their 5' or 3' flanking regions. Through the use of 5' and 3' specific cDNA fragments to probe the RNA fragments resulting from oligonucleotide directed cleavage by RNase H it was demonstrated that the two synapsin mRNAs have common 5', but diverging 3' extensions. The cleavage directed by oligo II yielded a single 0.8 kb fragment detected by the 5' probe E₃, but two fragments of 2.6 and 3.7 kb after hybridization with 3' probe E₂. This is consistent with the 1 kb size difference between the two mRNA species, which therefore can be mapped to the 3' portion of the RNA molecules.

These findings were further substantiated by the use of another oligonucleotide (oligo I) to direct cleavage by RNase H. This experiment yielded fragments of 4.2 and 3.1 kb 3' to the cleavage site, confirming the location of the 1 kb size difference in the 3' portion of the synapsin mRNAs. Ultimately, these results strongly suggest common 5', but diverging 3' ends of the two mRNA species.

4.2.3 The synapsin cDNA clone pSyn5 represents a copy of the 3.4 kb RNA species

Another piece of information was provided by the RNase H results. The synapsin cDNA pSyn5 includes the 3' end of the mRNA from which it was copied, indicated by its poly(A) tail. This cDNA is therefore a complete copy except at the 5' end. The RNase H assay demonstrated that both mRNAs have a 5' extension of 800 bases with respect

to the oligo-cutting site. The synapsin cDNA clone pSyn5 misses therefore roughly 300 bases at its 5' end and thus represents a copy of the smaller 3.4 kb mRNA. This information was not previously available. DNA sequencing of clone pSyn5 suggested that the start of translation, indicated by an AUG codon, was found close to the 5' end of this clone (McCaffery and DeGennaro, 1986). Therefore the missing 300 upstream bases of pSyn5 probably correspond to the 5' untranslated region of the 3.4 kb mRNA. This appears unusually long by comparison with other cellular mRNAs which show on average 20 - 80 nucleotides of 5' untranslated sequences according to a compilation study of Kozak (1984). However, this finding is consistent with the results of Tsou et al. (1986), who observed 5' untranslated sequences up to 365 nt in length in a family of rat brain mRNAs and the report of 1000 bases of 5' untranslated sequence found in a cDNA encoding acetylcholinesterase of *Drosophila melanogaster* (Hall and Spierer, 1986). These observations favour the interpretation that a long 5' untranslated regions is a special characteristic of neuron-specific mRNAs.

4.2.4 Analysis on the genomic level: identification of an alternatively spliced exon

What is the importance of the results discussed above with respect to the organization of the synapsin gene? Do they provide information about its genomic arrangement and the pathway of RNA processing which gives rise to the mature synapsin mRNAs? The results of the RNase H assay allow several predictions to be made about the organization of the synapsin gene:

The 0.8 kb 5' fragment common to both mRNAs strongly suggests a single initiation site in the synapsin gene for the transcription of both mRNAs. The existence of closely clustered transcriptional start sites cannot be excluded, however, because the resolution of the agarose gel system is not sufficient to detect such small size

differences. Closely nested transcriptional start sites have been shown for instance in a family of rat brain mRNAs by Tsou et al. (1986) and for multiple mRNAs of human tyrosine hydroxylase by Grima et al. (1987). In these cases primer extension analysis was used to demonstrate small differences in the location of the transcriptional start sites. While the evidence for the existence of a single transcriptional start site in the synapsin gene appeared strong, the organisation of the structural gene needed further investigation.

In order to determine the localization of the sequences specific for the 4.5 kb mRNA within the synapsin gene, DNA restriction fragments from synapsin-specific genomic clones were hybridized against poly(A)⁺ RNA from 10-day-old rat brain. DNA fragments were specifically chosen from the 3' end of the synapsin gene for this analysis. A 740 bp Pvu II fragment was identified which selectively recognized the 4.5 kb synapsin mRNA species. This genomic fragment was found within the 3' portion of the synapsin gene. It is flanked by fragments hybridizing to both synapsin mRNAs. The identification of this 4.5 kb mRNA-specific genomic fragment provided direct evidence for an alternatively spliced exon, which gives rise to the extra 1000 bases in this mRNA species.

Moreover the detection of this 4.5 kb-specific exon confirmed the hypothesis which was suggested by the structural analysis on the mRNA level:

(i) The 1000 base size difference between the synapsin mRNAs was clearly mapped to their 3' portions by the RNase H assay. This finding is consistent with the 3' location of the exon-containing Pvu II fragment within the synapsin gene (see fig. 23).

(ii) By comparison with the exon/intron structure of the synapsin gene, which has been partially established (Carroll, unpublished observation), it became obvious that the Pvu II-exon is inserted into the 4.5 kb mRNA within the region represented by fragment E₂ of pSyn5.

Its precise location was determined to be at the 3' end of the E₂ cDNA fragment (fig. 23).

(iii) After assessment of the high degree of homology between the two mRNA species with cDNA, it was proposed that insertion of additional sequences to produce the larger mRNA would most likely occur within the E₂ fragment. This proposal has now been verified by the identification and localization of the alternative Pvu II-exon (fig. 23).

Does this splicing event change the coding potential of the 4.5 kb mRNA? As shown in figure 23 the protein-coding region of the synapsin cDNA extends roughly 200 bases downstream from the preceeding identified 5' exon. According to genomic mapping, the insertion of the alternative exon may occur in this area or further downstream. There are no sequence data available yet which would define the exon/intron borders unambigously. Therefore it is not completely clear, whether or not the insertion of the extra exon into the 4.5 kb mRNA changes the carboxy terminus of the synapsin protein.

4.2.5 The 3' end of the synapsin gene is in sight

Another important piece of information has been gained from the hybridization of genomic probes with the synapsin mRNAs: the 3' border of the synapsin transcription unit can now be defined. Due to the localization of the extra 1000 bases of the 4.5 kb mRNA species to the internal Pvu II fragment, the most 3' exon-containing Eco RI fragment most likely represents the border of the synapsin gene. In fact no additional exons were found within a distance of 10 kb downstream of this Eco RI fragment, which contains exon(s) encoding the 3' end of both synapsin mRNAs (Carroll, unpublished observation). Taken together these findings strongly suggest that the 3' border of the synapsin gene can now be defined. Sequence analysis of this area of the gene will determine the exact location of motifs such as the

consensus sequence AATAAAA which is necessary for accurate cleavage and polyadenylation of the nascent RNA transcripts in eucaryotes (Proudfoot and Brownlee, 1976; Fitzgerald and Shenk, 1981).

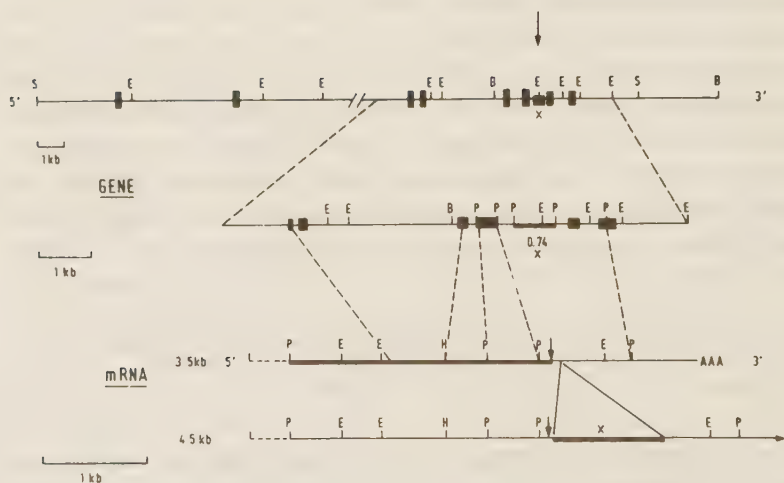


Fig. 23. Structural relationship of the synapsin gene and the two synapsin mRNA species.

Upper panel: restriction map of the synapsin gene with identified exons drawn as black boxes (not in scale). Arrow indicates location of alternative exon (X). Lower panel: restriction map of the synapsin cDNA (= 3.4 kb mRNA). 4.5 kb mRNA aligned below with putative position of the extra-sequence (X). Black bar indicates protein-coding region. Dotted line at 5' end of both mRNAs indicates their common 5' extension, shown by the RNase H assay.

4.2.6 An RNA processing model for the synapsin gene

Summarizing the data available about the organization of the synapsin gene a model for the RNA processing pathway can now be proposed:

The synapsin transcription unit has common start and stop signals. RNA diversity is created by alternative splicing only.

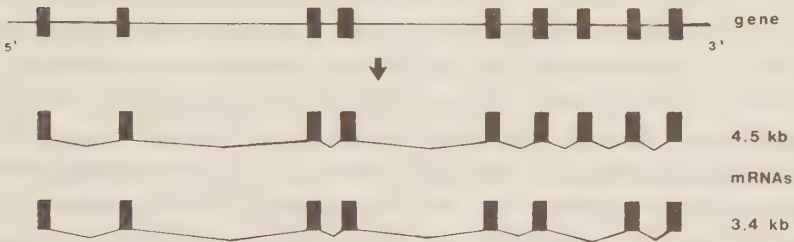


Fig. 24. RNA processing model of the synapsin transcription unit.

Schematic representation of the exon/intron structure of the synapsin gene. The alternative RNA splicing pattern is shown in the lower half, which is proposed to give rise to both synapsin mRNAs.

The evidence for this model can briefly be summarized as follows. The RNase H assay demonstrated that both mRNA species have the same 5' extension. This finding strongly suggests common (or very close) transcriptional start sites for both mRNAs species. Their characterization with different cDNA fragments provided evidence for common exons covering the whole cDNA sequence, including a common 3' end. This piece of evidence strongly argues for a single 3' cleavage and polyadenylation site. Finally the difference between the two mRNA species could be attributed to (an) alternatively spliced internal exons

within the 3' portion of the synapsin gene. The splicing mechanism seems to be regulated during neuronal development, because the 4.5 kb mRNA is only expressed in early stages of neuronal development, then it decreases to very low levels in comparison to the 3.4 kb mRNA (see section III).

The proposed RNA processing model for the synapsin gene can explain the macroheterogeneity of the 3.4 and 4.5 kb synapsin mRNA species. It does not, however, explain the microheterogeneity observed in synapsin mRNA (discussed in section III). The 3.4 kb mRNA species is thought to represent at least two different mRNA species of similar length. These transcripts are considered to encode the two synapsin proteins Ia and Ib. Evidence for such microheterogeneity has been provided by sequencing of the independent synapsin cDNA clone pSyn16 which showed a 40 base in-frame deletion in its 3' portion. This phenomenon of microheterogeneity is compatible with the proposed model, if one postulates the existence of one or several small alternatively spliced exons in the 3' protein-coding region.

Selective RNA production by alternative splicing only has been described previously for other cellular genes. The most prominent examples are the genes for human fibronectin (Schwarzbauer et al., 1984; Tamkun et al., 1984); for the c-Kirstin-ras-2-protooncogene (McGrath et al., 1983; Shimizu et al., 1983; Capon et al., 1983) and the preprotachykinin gene (Nawa et al., 1984). In these genes the selective inclusion of a particular exon changes the protein product derived from the differentially spliced mRNAs.

Most likely this is not the case for the synapsin gene. The major change caused by the inclusion of the alternative exon into the synapsin gene probably results in elongation of the 3' untranslated region of the 4.5 kb mRNA. Similar mRNA heterogeneity was found in the gene for mouse salivary and liver α -amylase (Hagenbuchle et

al., 1980; Young et al., 1981). α -amylase mRNA species specific for the salivary gland or liver differ in their 5' untranslated sequences. The mRNAs for the enzyme dehydrofolate reductase (DHFR) also show heterogeneity in their untranslated regions (Setzer et al., 1982). As many as seven mRNAs have been described which differ in their 3' ends due to the use of alternate polyadenylation sites. The protein-coding sequences in these mRNAs are not effected. What could be the reason for an RNA polymorphism which only effects the 5' or 3' untranslated region?

Data from sequence analysis predict an important function. Examination of 3' untranslated sequences of isotypic mRNAs of different species shows a stronger than expected conservation of nucleotide sequences and exon length (Miyata et al., 1980; Yaffe et al., 1985). This conservation was far greater than that seen between mRNAs for isoforms of proteins that are encoded by separate genes (Yaffe et al., 1985). Thus this conservation implies that 3' noncoding sequences are functionally important to gene expression. One possibility is that they interact with specific factors that affect RNA transport or stability. In the case of the c-fos proto-oncogene, the transformation potential is inhibited by 3' noncoding sequences that appears to interact with the carboxy terminal coding sequences to reduce translational or transport efficiencies of the c-fos mRNA (Miller et al., 1984). Indeed such sequences that might mediate mRNA transport or stability may be differentially recognized during development or in different tissues.

Alternative splicing of the primary synapsin RNA transcript might effect mRNA stability and result in the differential expression of both mRNA species seen during neuronal development.

4.2.7 Functional aspects of selective RNA production

An important functional aspect of selective RNA production from a single gene is to provide a means for eucaryotic systems to produce complexity in gene expression in either a developmental or tissue-specific fashion. This aspect may also concern the expression of the synapsin gene: The two synapsin mRNAs were found to be differentially regulated during neuronal development. It is possible that both mRNAs, found at different periods of cerebellar development, are expressed in different stages of neuronal differentiation. This question can now be approached by studying the expression of the 4.5 kb mRNA species separately. Through the identification of the alternative exon within the Pvu II DNA fragment a specific tool is now available to localize the 4.5 kb mRNA individually in neuronal tissue by *in situ* hybridization. The expression of the 4.5 kb mRNA in different subtypes of neurons compared to the 3.4 kb synapsin mRNA (which has been localized in granule cells of the cerebellum undergoing synaptogenesis) can now be elucidated.

In addition, the coding potential of the 4.5 kb mRNA class can now be directly tested by hybrid selection experiments. The 4.5 kb specific genomic sequences can be used to select the 4.5 kb mRNA from a total poly(A)⁺ RNA population by hybridization. The selected mRNA species can then be translated *in vitro* and the protein products identified by synapsin specific antibodies. This will answer the as yet unresolved question as to the exact relationship between the two mRNAs and the two protein species.

Ultimately this study represents one of the first investigations of the regulation of gene expression for a neuron-specific protein. This work makes possible the study of the role of RNA processing in the creation of diversity of gene expression and determining neuronal phenotype.

V. REFERENCES

- Akam, M.E. (1983) The location of Ultrabithorax transcript in *Drosophila* tissue sections. *EMBO J.*, **2**, 2075-2084.
- Alonso, C. (1973) Improved conditions for *in situ* RNA/DNA hybridization. *FEBS Lett.*, **31**, 85-88.
- Alt, F.W., Bothwell, A.L.M., Knapp, M., Siden, E. Mather, E., Koshland, M. and Baltimore, D. (1980) Synthesis of secreted and membrane-bound immunoglobulin Mu heavy chains is directed by mRNAs that differ at their 3' ends. *Cell*, **20**, 293-301.
- Altman, J. 1972a. Postnatal development of the cerebellar cortex in the rat. I. The external germinal layer and the transitional molecular layer. *J. Comp. Neur.*, **145**, 353-398.
- Altman, J. 1972b. Postnatal development of the cerebellar cortex in the rat. II. Phases in the maturation of Purkinje cells and of the molecular layer. *J. Comp. Neur.*, **145**, 399-464.
- Altman, J. 1972c. Postnatal development of the cerebellar cortex in the rat. III. Maturation of the components of the granular layer. *J. Comp. Neur.*, **145**, 465-513.
- Amara, S.G., Jonas, V., Rosenfeld, M.G., Ong, E.S., Evans, R.M. (1982) Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. *Nature*, **298**, 240-244.
- Angerer, L.M. and Angerer, R.C. (1981) Detection of poly(A)⁺ RNA in sea urchin eggs and embryos by quantitative *in situ* hybridization. *Nucleic Acids Res.*, **9**, 2819-2840.
- Aviv, H. and Leder, P. (1972) Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. *Proc. Natl. Acad. Sci. USA*, **69**, 1408-1412.
- Bähler, M. and Greengard, P. (1987) Synapsin I bundles F-actin in a phosphorylation-dependent manner. *Nature*, **326**, 704-707.
- Baines, A.J. and Bennett, V. (1985) Synapsin I is a spectrin-binding protein immunologically related to erythrocyte protein 4.1. *Nature*, **315**, 410-413.
- Baines, A.J. and Bennett, V. (1986) Synapsin I is a microtubule-bundling protein. *Nature*, **319**, 145-147.

Bantle, J.A. and Hahn, W.E. (1976) Complexity and characterization of polyadenylated RNA in mouse brain. *Cell*, **8**, 139-150.

Bandtlow, C.E., Heumann, R., Schwab, M.E. and Thoenen, H. (1987) Cellular localization of nerve growth factor synthesis by *in situ* hybridization. *EMBO J.*, **4**, 891-899.

Bennett, M.K., Erondy, N.E. and Kennedy, M.B. (1983) Purification and characterization of a calmodulin-dependent protein kinase that is highly concentrated in brain. *J. Biol. Chem.*, **258**, 12735-12740.

Berger, C.N. (1986) *In situ* localization of immunoglobulin-specific RNA in single cells of the B lymphocyte lineage with radiolabelled probe. *EMBO J.*, **5**, 85-93.

Berkower, I., Leis, J. and Hurwitz, J. (1973). Isolation and characterization of an endonuclease from *Escherichia coli* specific for ribonucleic acid in ribonucleic acid-deoxyribonucleic acid hybrid structures. *J. Biol. Chem.*, **248**, 5914-5921.

Biguët, N.F., Buda, M., Lamouroux, A., Samolyk, D. and Mallet, J. (1986) Time course of the changes of TH mRNA in rat brain and adrenal medulla after a single injection of reserpine. *EMBO J.*, **5**, 287-291.

Birnboim, H.C. and Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant DNA. *Nucleic Acids. Res.*, **7**, 1513-1523.

Bloch, B., Milner, R.J., Baird, A., Gubler, U., Reymond, C.H., Bollen, P., Le Guellec, D., Bloom, F.E. (1984) Detection of the messenger RNA coding for prepro-enkephalin A in bovine adrenal by *in situ* hybridization. *Regul. Pept.*, **8**, 345-354.

Bloom, F.E., Ueda, T. and Greengard, P. (1979) Immunocytochemical localization in synapses of Protein I, an endogenous substrate for protein kinases in mammalian brain. *Proc. Natl. Acad. Sci. USA*, **76**, 5982-5986.

Brahic, M. and Haase, A.T. (1978) Detection of viral sequences of low reiteration frequency by " *in situ* " hybridization. *Proc. Natl. Acad. Sci. USA*, **75**, 6125-6129.

Branks, P.L. and Wilson, M.C. (1986) Patterns of gene expression in the murine brain revealed by *in situ* hybridization of brain-specific mRNAs. *Mol. Brain Res.*, **1**, 1-16.

Butler, E.T. and Chamberlin, M.J. (1982) Bacteriophage SP6-specific RNA polymerase. I. Isolation and characterization of the enzyme. *J. Biol. Chem.*, **257**, 5772-5778.

Capon, D.J., Seeburg, P.H., McGrath, J.P., Hayflick, J.S., Edman, U., Levinson, D. and Goeddel, D.V. (1983)

Activation of Ki-ras 2 gene in human colon and lung carcinomas by two different point mutations. *Nature*, **304**, 507-512.

Chien-Tsung Hu, M., Davidson, N. (1986) Mapping transcriptional start points on cloned genomic DNA with T4 DNA polymerase: a precise and convenient technique. *Gene*, **42**, 21-29.

Chikaraishi, D.M. (1979) Complexity of cytoplasmic polyadenylated and nonpolyadenylated rat brain RNAs. *Biochemistry*, **18**, 3249-3256.

Cotman, C.W. and Nieto-Sampedro, M. (1984) Cell biology of synaptic plasticity. *Science*, **225**, 1287-1294.

Cox, K.H., De Leon, D.V., Angerer, L.M., and Angerer, R.C. (1984) Detection of mRNAs in sea urchin embryos by *in situ* hybridization using asymmetric RNA probes. *Dev. Biol.*, **101**, 485-502.

Darnell, J. (1982) Variety in the level of gene control in eucaryotic cells. *Nature*, **297**, 365-371.

De Camilli, P., Ueda, T., Bloom, F.E., Battenberg, E. and Greengard, P. (1979) Widespread distribution of Protein I in the central and peripheral nervous system. *Proc. Natl. Acad. Sci. USA*, **76**, 5977-5981.

De Camilli, P., Cameron, R. and Greengard, P. (1983a) Synapsin I (Protein I), a nerve terminal-specific phosphoprotein. I. Its general distribution in synapses of the central and peripheral nervous system demonstrated by immunofluorescence in frozen and plastic sections. *J. Cell Biol.*, **96**, 1209-1211.

DeCamilli, P., Harris, S.M., Huttner, W.B. and Greengard, P. (1983b) II. Synapsin I (Protein I), a nerve terminal-specific phosphoprotein. Its specific association with synaptic vesicles demonstrated by immunocytochemistry in agarose-embedded synaptosomes. *J. Cell Biol.*, **96**, 1209-1211.

DeGennaro, L.J., Kanazir, S.D., Wallace, W.C., Lewis, R.M. and Greengard, P. (1983) Neuron-specific phosphoproteins as models for neuronal gene expression. *Cold Spring Harbor Symp. Quant. Biol.*, **48**, 337-345.

De Martinville, B., Wyman, A.R., White, R. and Francke, U. (1982) Assignment of the first random restriction fragment length polymorphism (RFLP) locus (D14S1) to a region of human chromosome 14. *Am. J. Hum. Genet.*, **34**, 216-226.

Dolphin, A.C. and Greengard, P. (1981) Serotonin stimulates phosphorylation of Protein I in the facial motor nucleus of rat brain. *Nature*, **289**, 76-79.

Donis-Keller, H. (1979) Site-specific enzymatic cleavage of RNA. *Nucleic Acids Res.*, **7**, 179-192.

Early, P., Rogers, J., Davis, M., Calame, K., Bond, M., Wall, R. and Hood, L. (1980) Two mRNAs can be produced from a single immunoglobulin μ gene by alternative RNA processing pathways. *Cell*, **20**, 313-319.

Eiden, L.E., Giraud, P., Affolter, H., Herbert, E. and Hotchkiss, A.J. (1984) Alternative modes of enkephalin biosynthesis regulation by reserpine and cyclic AMP in cultured chromaffin cells. *Proc. Natl. Acad. Sci. USA*, **81**, 3949-3953.

Ellis, L., Katz, F. and Pfenninger, K.H. (1985) Nerve growth cones isolated from fetal rat brain. II. Cyclic adenosine 3':5'-monophosphate (cAMP)-binding proteins and cAMP-dependent protein phosphorylation. *J. Neurosci.* **5**, 1393-1401.

Favaloro, J., Freismann, R., Kamen, R. (1980) Transcription maps of polyoma virus specific RNA analysis by two-dimensional nuclease S1 gel mapping. *Methods Enzymol.*, **65**, 718-749.

Feinberg, A.P. and Vogelstein, B. (1984) A technique of radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.*, **132**, 6-13. Addendum to this publication: *Anal. Biochem.*, **137**, 266-267.

Fitzgerald, M. and Shenk, T. (1981) The sequence 5'-AAUAAA-3' forms part of the recognition site for polyadenylation of late SV40 mRNAs. *Cell*, **24**, 251-260.

Forn, J. and Greengard, P. (1978) Depolarizing agents and cyclic nucleotides regulate the phosphorylation of specific neuronal proteins in rat cerebral cortex slices. *Proc. Natl. Acad. Sci. USA*, **75**, 5195-5199.

Fried, G., Nestler, E.J., DeCamilli, P., Stjärne, L., Olsen, L., Lundberg, J.M., Hökfelt, T., Ouimet, C.C. and Greengard, P. (1982) Cellular and subcellular localization of Protein I in the peripheral nervous system. *Proc. Natl. Acad. Sci. USA*, **79**, 2717-2721.

Gal, A., Stolzenberger, C., Wienker, T., Wieacker, P., Ropers, H., Friedrich, U., Bleeker-Wagemakers, L., Pearson, P. and Warburg, M. (1985) Norries disease - close linkage with genetic markers from the proximal short arm of the X-chromosome. *Clin. Genet.*, **27**, 282-283.

Gall, J.G. and Pardue, M.L. (1969) Formation and detection of RNA-DNA hybrid molecules in cytological preparations. *Proc. Natl. Acad. Sci. USA*, **64**, 600-604.

Gee, C.E. and Roberts, J.L. (1983) "In situ" hybridization histochemistry: A technique for the study of gene expression in single cells. *DNA*, **2**, 155-163.

Gee, C.H., Chen, C.L., Roberts, J.L., Thompson, R. and Watson, S.J. (1983) Identification of proopiomelanocortin neurons in rat hypothalamus by "in situ" cDNA-mRNA hybridization. *Nature*, **306**, 374-376.

Gilgenkranz, S., Tridon, P., Pinel-Briquet, N., Beurey, J. and Weber, M. (1985) Translocation (X-9)(P11-Q34) in a girl with incontinentia pigmenti (IP) - implications for the regional assignment of the IP locus to XP11. *Hum. Genet.*, **28**, 90-92.

Ginzburg, I., Teichmann, A., Griffin, W.S.T. and Littauer, V.Z. (1986) Differential expression of α -tubulin mRNA in rat cerebellum as revealed by in situ hybridization. *FEBS Lett.*, **194**, 161-164.

Glisin, V., Crkvenjakov, R. and Byus, C. (1974) Ribonucleic acid isolated by cesium chloride centrifugation. *Biochemistry*, **13**, 2633-2637.

Goelz, S.E., Nestler, E.J. and Greengard, P. (1985) Phylogenetic survey of proteins related to synapsin I and biochemical analysis of four such proteins from fish brain. *J. Neurochem.*, **45**, 63-72.

Goelz, S.E., Nestler, E.J., Chehraz, B. and Greengard, P. (1981) Distribution of Protein I in mammalian brain as determined by a detergent-based radioimmunoassay. *Proc. Natl. Acad. Sci. USA*, **78**, 2130-2134.

Goldenring, J.R., Lasher, R.S., Vallano, M.L., Ueda, T., Naito, S., Sternberger, N.H., Sternberger, L.A. and De Lorenzo, R.J. (1986) Association of synapsin I with neuronal cytoskeleton. Identification in cytoskeletal preparations in vitro and immunocytochemical localization in brain of synapsin I. *J. Biol. Chem.*, **261**, 8495-8504.

Goodfellow, P.N., Davies, K.E. and Ropers, H. (1985) Report of the committee on the genetic constitution of the X-chromosomes and Y-chromosomes. *Cytogenet. Cell. Genet.*, **40**, 296-352.

Goodman, S.R. and Zagon, I.S. (1986) The neural cell spectrin skeleton: a review. *Am. J. Physiol.*, **250**, 347-360.

Gottlieb, D.J. and Glaser, L. (1975) A novel assay of neuronal cell adhesion. *Biochem. Biophys. Res. Commun.*, **63**, 815-821.

Green, M.R., Maniatis, T. and Melton, D.A. (1983) Human β globin pre-mRNA synthesized in vitro is accurately spliced in *Xenopus* oocyte nuclei. *Cell*, **32**, 681-694.

Griffin, W.S.T., Alejos, M.A., Cox, E.J. and Morrison, M.R. (1985) The differential distribution of β -tubulin mRNAs in individual mammalian brain cells. *J. Cell. Biochem.*, **27**, 205-214.

Griffin, W.S.T. and Morrison, M.R. (1985) *In situ* hybridization - Visualisation and quantitation of genetic expression in mammalian brain. *Peptides*, **6**, 89-96.

Grima, B., Lamairoux, A., Boni, C., Julien, J.-F., Iavoy-Agid, F., Mallet, J. (1987) A single human gene encoding multiple tyrosine hydroxylases with different predicted functional characteristics. *Nature*, **326**, 707-711.

Grummt, J., Soellner, C. and Scholz, I. (1979) Structural organisation of mouse rDNA: comparison of transcribed and non-transcribed regions. *Nucleic Acids Res.*, **6**, 1351-1369.

Gubler, V. and Hoffmann, B.J. (1983) A simple and very efficient method for generating cDNA libraries. *Gene*, **25**, 263-269.

Haberkern, R.C. and Cantoni, G.L. (1973) Studies on a calf thymus ribonuclease specific for ribonucleic acid-deoxyribonucleic acid hybrids. *Biochemistry*, **12**, 2389-2395.

Hafen, E., Kurviwa, A. and Gehring, W.J. (1984) Spatial distribution of transcript from the segmentation gene *fushi tarazu* during *Drosophila* embryonic development. *Cell*, **37**, 833-841.

Hafen, E., Levine, M., Garber, R.L. and Gehring, W.J. (1983) An improved *in situ* hybridization method for the detection of cellular RNAs in *Drosophila* tissue sections and its application for localizing transcript of the homeotic *Antennapedia* gene complex. *EMBO J.*, **2**, 617-623.

Hagenbuchle, O., Tosi, M., Schibler, U., Bovey, R., Wellauer, P.K. and Young, R.A. (1981) Mouse liver and salivary gland α -amylase mRNAs differ only in 5' non-translated sequences. *Nature*, **289**, 643-646.

Hall, M.C. and Spierer, P. (1986) The *Ace* locus of *Drosophila melanogaster*: structural gene for acetylcholinesterase with an unusual 5' leader. *EMBO J.*, **5**, 2949-2954.

Harper, M.E., Ullrich, A., Saunders, G.F. (1981) Localization of the human insulin gene to the distal end of short arm of chromosome 11. *Proc. Natl. Acad. Sci. USA*, **78**, 4458-4460.

Harrison, P.R., Conrie, D., Paul, J., Jones, K.W. (1973) Localization of cellular globin messenger RNA by "in situ" hybridization to complementary DNA. *FEBS Lett.*, **32**, 4458-4460.

Hayashi, S., Gillam, I.C., Delaney, A.D. and Tener, G.M. (1978) Acetylation of chromosome squashes of *Drosophila melanogaster* decreases the background in autoradiographs from hybridization with (125 J)-labeled RNA. *J. Histochem. Cytochem.*, **36**, 677-679.

Hecht, R.M., Gossett, L.A. and Jeffery, W.R. (1981) Ontogeny of maternal and newly transcribed mRNA analysed by *in situ* hybridization during development of *Caenorhabditis elegans*. *Dev. Biol.*, **83**, 374-379.

Henry, C.M., Ferdinand, F.J. and Knippers, R. (1973) A hybridase from *Escherichia coli*. *Biochem. Biophys. Res. Comm.*, **50**, 603-611.

Hodgson, S.V., Neville, B., Iones, R.W.A., Fear, C. and Bobrow, M. (1985) Two cases of X/autosome translocation in females with incontinentia pigmenti. *Hum. Genet.*, **71**, 231-234.

Hubel, D.H. (1979) The brain. *Sci. Am.*, **241**, 44-53.

Hudson, P., Penschow, J., Shine, J., Ryan, G., Niall, H. and Coghlan, J. (1981) Hybridization histochemistry: use of recombinant DNA as a "homing probe" for tissue localization of specific mRNA populations. *Endocrinology*, **108**, 353-356.

Huttner, W.B., Schiebler, W., Greengard, P. and DeCamilli, P. (1983) Synapsin I (Protein I), a nerve terminal-specific phosphoprotein. III. Its association with synaptic vesicles studied in a highly purified synaptic vesicle preparation. *J. Cell Biol.*, **96**, 1374-1388.

Huttner, W.B., DeGennaro, L.J. and Greengard, P. (1981) Differential phosphorylation of multiple sites in purified Protein I by cyclic AMP-dependent and calcium-dependent protein kinases. *J. Biol. Chem.*, **256**, 1482-1488.

Huttner, W.B. and Greengard, P. (1979) Multiple phosphorylation sites in Protein I and their differential regulation by cyclic AMP and calcium. *Proc. Natl. Acad. Sci. USA*, **75**, 5402-5406.

Jakobs, J.W., Simpson, E., Penschow, P., Hudson, F., Coghlan, J. and Niall, H. (1983) Characterization and localization of calcitonin messenger ribonucleic acid in rat thyroid. *Endocrinology*, **113**, 1616-1622.

John, H.A., Birnstiel, M.L. and Jones, K.W. (1969) RNA-DNA hybrids at the cytological level. *Nature*, **223**, 582-587.

Johnson, E.M., Ueda, T., Maeno, H. and Greengard, P. (1972) Adenosine 3',5'-monophosphate-dependent phospho-

rylation of a specific protein in synaptic membrane fractions from rat cerebrum. *J. Biol. Chem.*, **247**, 5650-5652.

Kassavetis, G.A., Butler, E.T., Roulland, D. and Chamberlin, M.J. (1982) Bacteriophage SP6-specific RNA polymerase. II. Mapping of SP6 DNA and selective *in vitro* transcription. *J. Biol. Chem.*, **257**, 5779-5788.

Kennedy, M.B. and Greengard, P. (1981) Two calcium/calmodulin-dependent protein kinases, which are highly concentrated in brain, phosphorylate Protein I at distinct sites. *Proc. Natl. Acad. Sci. USA*, **78**, 1293-1297.

Kennedy, M.B., McGuinness, T. and Greengard, P. (1983) A calcium/calmodulin-dependent protein kinase activity from mammalian brain that phosphorylates Synapsin I: Partial purification and characterization. *J. Neurosci.*, **3**, 818-831.

Kilimann, M.W. and DeGennaro, L.J. (1985) Molecular cloning of cDNAs for the nerve-cell specific phosphoprotein, synapsin I. *EMBO J.*, **4**, 1997-2002.

Kozak, M. (1980) Compilation and analysis of sequences upstream from the translational start sites in eucaryotic mRNAs. *Nucleic Acids Res.*, **12**, 1067-1082.

Krieg, P.A. and Melton, D.A. (1984) Formation of the 3' end of histone mRNA by post-translational processing. *Nature*, **308**, 203-206.

Krueger, B.K., Forn, J. and Greengard, P. (1977) Depolarization induced phosphorylation of specific proteins, mediated by calcium ion influx, in rat brain synaptosomes. *J. Biol. Chem.*, **251**, 2764-2773.

Lazarides, E. and Noon, R.T. (1984) Assembly and topogenesis of the spectrin-based membrane skeleton in erythroid development. *Cell*, **37**, 354-356.

Leff, S.E., Rosenfeld, M.G. and Evans, R.M. (1986) Complex transcriptional unit: diversity in gene expression by alternative RNA processing. *Annu. Rev. Biochem.*, **55**, 1091-1170.

Llinas, R., McGuinness, T.L., Leonard, C.S., Sugimori, M. and Greengard, P. (1985) Intraterminal injection of synapsin I or calcium/calmodulin-dependent protein kinase II alters neurotransmitter release at the squid giant synapse. *Proc. Natl. Acad. Sci. USA*, **82**, 3035-3039.

Lohmann, S.M., Ueda, T. and Greengard, P. (1978) Ontogeny of synaptic proteins in brain. *Proc. Natl. Acad. Sci. USA*, **75**, 4037-4041.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, N.Y.

Mason, C.A. (1987) Axon development in mouse cerebellum: embryonic axon forms and expression of synapsin I. *Neuroscience*, **19**, 1319-1333.

McAllister, L.B., Scheller, R.H., Kandel, E.R. and Axel, R. (1983) *In situ* hybridization to study the origin and fate of identified neurons. *Science*, **222**, 800-808.

McCabe, J.T., Morell, J.I., Ivell, R., Schmale, H., Richter, D. and Pfaff, D.W. (1986) *In situ* hybridization technique to localize rRNA and mRNA in mammalian neurons. *J. Histochem. Cytochem.*, **34**, 45-50.

McCaffery, C.A. and DeGennaro, L.J. (1986). Determination and analysis of the primary structure of the nerve terminal specific phosphoprotein, synapsin I. *EMBO J.*, **5**, 3167-3173.

McGrath, J.P., Capon, D.J., Smith, D.H., Chen, E.Y., Seeburg, P.H., Goeddel, D.V. and Levinson, A.D. (1983) Structure and organization of the human ki-ras proto-oncogene and a related processed pseudogene. *Nature*, **304**, 501-507.

McGuinness, T.L., Lai, Y. and Greengard, P. (1985) Ca^{2+} /calmodulin-dependent protein kinase. II. Isozymic forms from rat forebrain and cerebellum. *J. Biol. Chem.*, **260**, 1696-1704.

Melton, D.A., Krieg, P.A., Rebgliati, M.R., Maniatis, T., Zinn, K. and Green, M.R. (1984) Efficient *in vitro* synthesis of biologically active RNA and RNA probes from plasmids containing a bacteriophage SP6 promotor. *Nucleic Acids. Res.*, **13**, 1-13.

Miller, A.D., Curran, T. and Verma, I.M. (1984) c-fos protein can induce cellular transformation: A novel mechanism of activation of a cellular oncogene. *Cell*, **36**, 51-60.

Milner, R.J. and Sutcliffe, J.G. (1983) Gene expression in rat brain. *Nucleic Acids Res.*, **11**, 5497-5520.

Miyata, T., Yasunaga, T. and Nishida, T. (1980) Nucleotide sequence divergence and functional constraint in mRNA evolution. *Proc. Natl. Acad. Sci. USA*, **77**, 7328-7332.

Moar, M.H., Purdom, I.F. and Jones, K.W. (1975) Influence of temperature on detectability and chromosomal distribution of specific DNA sequences by *in situ* hybridization. *Chromosoma (Berl.)*, **53**, 345-359.

Mobley, P. and Greengard, P. (1985) Evidence for widespread effects of noradrenaline on axon terminals in the rat frontal cortex. *Proc. Natl. Acad. Sci. USA*, **82**, 945-947.

Navone, F., Greengard, P. and De Camilli, P. (1984) Synapsin in nerve terminals: selective association with small synaptic vesicles. *Science*, **226**, 1209-1211.

Nawa, H., Kotani, H. and Nakanishi, S. (1984) Tissue-specific generation of two preprotachykinin mRNAs from one gene by alternative RNA splicing. *Nature*, **312**, 729-734.

Nestler, E.J. and Greengard, P. (1982a) Nerve impulses increase the state of phosphorylation of Protein I in the rabbit superior cervical ganglion. *Nature*, **296**, 452-454.

Nestler, E.J. and Greengard, P. (1982b) Distribution of Protein I and regulation of its state of phosphorylation in the rabbit superior cervical ganglion. *J. Neurosci.*, **2**, 1011-1023.

Nestler, E.J. and Greengard, P. (1980) Dopamine and depolarizing agents regulate the state of phosphorylation of Protein I in mammalian superior cervical sympathetic ganglion. *Proc. Natl. Acad. Sci. USA*, **77**, 7479-7483.

Ngai, J., Stack, J.H., Moon, R.T. and Lazarides, E. (1987) Regulated expression of multiple chicken erythroid membrane skeletal protein 4.1 variants is governed by differential RNA processing and translational control. *Proc. Nat. Acad. Sci. USA* **84**, 4432 - 4436.

Okayama, H. and Berg, P. (1982) High-efficiency cloning of full-length cDNA. *Mol. Cell. Biol.*, **2**, 161-170.

Orth, G., Jeanteur, P. and Croissant, O. (1970) Evidence for and localization of vegetative viral DNA replication by autoradiographic detection of RNA-DNA hybrids in sections of tumors induced by Shope papilloma virus. *Proc. Natl. Acad. Sci. USA*, **68**, 1876-1880.

Owens, G.P., Chaudhari, N. and Hahn, W.E. (1985) Brain "identifier sequence" is not restricted to brain: similar abundance in nuclear RNA in other organs. *Science*, **229**, 1263-1265.

Pochet, R., Brocas, H., Vassart, G., Toubeau, G., Seo, H., Refetoff, S., Dumont, J.E. and Pasteels, J.L. (1981) Radioautographic localization of prolactin messenger RNA on histological sections by *in situ* hybridization. *Brain Res.*, **211**, 433-438.

Proudfoot, N.J. and Brownlee, G.G. (1976) 3' non-coding region sequences in eucaryotic mRNA. *Nature*, **263**, 211-214.

Rigby, P.W.J., Dieckmann, M., Rhodes, C. and Berg, P. (1977) Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase. *J. Mol. Biol.*, **113**, 237-251.

Rogers, A.W. (ed.) (1979) Techniques in autoradiography. Biochemical Press, Amsterdam.

Rogers, J., Early, P., Carter, C., Calame, K., Bond, M., Hood, L. and Wall, R. (1980) Two mRNAs with different 3' ends encode membrane-bound and secreted forms of immunoglobulin μ chain. *Cell*, **20**, 303-312.

Rosenfeld, M.G., Roos, B.A., Ong, E.S. and Evans, R.M. (1981) Altered expression of the calcitonin gene associated with RNA polymorphism. *Nature*. **290**, 63-65.

Rosenfeld, M.G., Lin, C.R., Amara, S.G., Stolarsky, L., Roos, B.A., Ong, E.S. and Evans, R.M. (1982) Calcitonin mRNA polymorphism: Peptide switching associated with alternative RNA splicing events. *Proc. Natl. Acad. Sci. USA*, **79**, 1717-1721.

Ruppert, C., Goldowitz, D. and Wille, W. (1986) Proto-oncogene *c-myc* is expressed in cerebellar neurons at different developmental stages. *EMBO J.*, **5**, 1897-1901.

Sapienza, C. and St-Jacques, B. (1986) "Brain-specific" transcription and evolution of the identifier sequence. *Nature*, **319**, 418-420.

Schalling, M., Hökfelt, T., Wallace, B., Goldstein, M., Filer, D., Yamin, C. and Schlesinger, D.H. (1986) Tyrosin-3-hydroxylase in rat brain and adrenal medulla: hybridization histochemistry and immunohistochemistry combined with retrograde tracing. *Proc. Natl. Acad. Sci. USA*, **83**, 6208-6212.

Schiebler, W., Jahn, R., Doucet, J.P., Rothlein, J. and Greengard, P. (1986) Characterization of synapsin I binding to small synaptic vesicles. *J. Biol. Chem.*, **261**, 8383-8390.

Schwarzbauer, J.E., Tamkun, J.W., Lenischka, I.R. and Hynes, R.O. (1983) Three different fibronectin mRNAs arise by alternative splicing within the coding region. *Cell*, **35**, 421-431.

Scott Young, III, W., Mezey, E. and Siegel, R.E. (1986) Vasopressin and oxytocin mRNA in adrenalectomized and Brattleboro rats analysis by quantitative *in situ* hybridization histochemistry. *Mol. Brain Res.*, **1**, 231-241.

Setzer, D.R., McGrogan, M. and Schimke, R.T. (1982) Nucleotide sequence surrounding multiple polyadenylation sites in the mouse dihydrofolate reductase gene. *J. Biol. Chem.*, **257**, 5143-5147.

Shaw, P., Sordat, B. and Schibler, U. (1986) Developmental coordination of α -amylase and PSP gene expression during mouse parotid gland differentiation is controlled posttranscriptionally. *Cell*, **47**, 107-112.

Shimizu, K., Birnbaum, D., Ruley, M.A., Fasano, O., Suard, Y., Edlund, L., Taporsky, E., Goldfarb, M. and Wigler, M. (1983) Structure of the Ki-ras gene of the human lung carcinoma cell line Calu-1. *Nature*, **304**, 497-500.

Shivers, B.D., Harlan, R.E., Pfaff, D.W. and Schachter, B.S. (1986) Combination of immunocytochemistry and *in situ* hybridization in the same tissue section of rat pituitary. *J. Histochem. Cytochem.* **34**, 39-46.

Sieghart, W., Forn, J., Schwarz, R., Coyle, J.T. and Greengard, P. (1978) Neuronal localization of specific brain phospho-proteins. *Brain Res.*, **165**, 345-350.

Sieghart, W., Forn, J. and Greengard, P. (1979) Ca^{2+} and cyclic AMP regulate phosphorylation of the same two membrane-associated proteins specific to nervous tissue. *Proc. Natl. Acad. Sci. USA*, **76**, 2475-2479.

Singh, L. and Jones, K.W. (1984) The use of heparin as a simple cost-effective means of controlling background in nucleic acid hybridization procedures. *Nucl. Acids Res.* **12**, 5627-5638.

Singh, L., Purdom, J.F. and Jones, K.W. (1977) Effect of different denaturing agents on detectability of specific DNA sequences of various base compositions by *in situ* hybridization. *Chromosoma (Berl.)*, **60**, 377-389.

Sippel, A.E., Stavrianopoulos, J.G., Schutz G. and Feigelson, P. (1974) Translational properties of rabbit globin mRNA after specific removal of poly(A) with ribonuclease H. *Proc. Natl. Acad. Sci. USA*, **71**, 4635-4639.

Sorensen, R.G. and Babitch, J.A. (1984) Identification and comparison of Protein I in chick and rat forebrain. *J. Neurochem.*, **42**, 705-710.

Stavrianopoulos, J.G. and Chargaff, E. (1973) Purification and properties of ribonuclease H of calf thymus. *Proc. Natl. Acad. Sci. USA*, **70**, 1959-1963.

Strombom, U., Forn, J., Dolphin, A.C. and Greengard, P. (1979) Regulation of the state of phosphorylation of specific neuronal proteins in mouse brain by *in vivo* administration of anesthetic and convulsant agents. *Proc. Natl. Acad. Sci. USA*, **76**, 4687-4690.

Sutcliffe, J.G., Milner, R.J., Gottesfeld, J.M. and Lerner, R.A. (1984) Identifier sequences are transcribed specifically in brain. *Nature*, **308**, 237-241.

Tamkun, J.W., Schwarzbauer, J.E. and Hynes, R.O. (1984) A single fibronectin gene generates three different mRNAs by alternative splicing of a complex exon. *Proc. Natl. Acad. Sci. USA*, **81**, 5140-5144.

Tapprich, W.E. and Hill, W.E. (1986) Involvement of bases 787-795 of *Escherichia coli* 16 S ribosomal RNA in ribosomal subunit association. *Proc. Natl. Acad. Sci. USA*, **83**, 556-560.

Thomas, P.S. (1980) Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA*, **77**, 5201-5205.

Tsou, K. and Greengard, P. (1982) Regulation of phosphorylation of Proteins I, IIIa, and IIIb in rat neurohypophysis *in vitro* by electrical stimulation and by neuroactive agents. *Proc. Natl. Acad. Sci. USA*, **79**, 6075-6079.

Tsou, A., Lai, C., Danielson, P., Noonan, D.J. and Sutcliffe, J.G. (1986) Structural characterization of a heterogenous family of rat brain mRNAs. *Mol. Cell. Biol.*, **6**, 768-778.

Ueda, T., Maeno, H. and Greengard, P. (1973) Regulation of endogenous phosphorylation on specific proteins in synaptic membrane fractions from rat brain by adenosine 3':5'-monophosphate. *J. Biol. Chem.*, **248**, 8295-8305.

Ueda, T. and Greengard, P. (1977) Adenosine 3':5'-monophosphate regulated phosphoprotein system of neuronal membranes. I. Solubilization, purification and some properties of an endogenous phosphoprotein. *J. Biol. Chem.*, **252**, 5155-5163.

Ueda, T., Greengard, P., Berzins, K., Cohen, R.S., Blomberg, F., Grab, D.J. and Siekevitz, P. (1979) Subcellular distribution in cerebral cortex of two proteins phosphorylated by cAMP-dependent protein kinase. *J. Cell Biol.*, **83**, 308-319.

Uhl, G.R., Zingg, H.H. and Habener, J.F. (1985) Vasopressin mRNA *in situ* hybridization: Localization and regulation studied with oligonucleotide cDNA probes in normal and Brattleboro rat hypothalamus. *Proc. Natl. Acad. Sci. USA*, **82**, 5555-5559.

Ullrich, A., Shine, I., Chirgwin, J., Pictet, R., Tischer, E., Rutter, W.J. and Goodman, H.M. (1977) Rat insulin genes: Construction of plasmids containing the coding sequences. *Science*, **196**, 1312-1319.

Van Ness, J., Maxwell, I.H. and Hahn, W.E. (1979) Complex population of nonpolyadenylated messenger RNA in mouse brain. *Cell*, **18**, 1341-1349.

Vournakis, J.N., Efstratiadis, A. and Kafatos, F.C. (1975) Electrophoretic patterns of deadenylated chorion and globin mRNAs. *Proc. Natl. Acad. Sci. USA*, **72**, 2959-2963.

Worley, P.F., Baraban, J.M., De Souza, E.B., Synder, S. (1986) Mapping second messenger system in the brain: Differential localization of adenylate cyclase and protein kinase C. *Proc. Natl. Acad. Sci. USA*, **83**, 4053-4057.

Wuenschell, C.W., Fisher, R.S., Kaufman, D.L. and Tobin, A.J. (1986) *In situ* hybridization to localize mRNA encoding the neurotransmitter synthetic enzyme glutamate decarboxylase in mouse cerebellum. *Proc. Natl. Acad. Sci. USA*, **83**, 6193-6197.

Yaffe, D., Nudel, U., Mayer, Y. and Neumann, S. (1985) Highly conserved sequences in the 3' untranslated region of mRNAs coding for homologous proteins in distantly related species. *Nucleic Acids Res.*, **13**, 7232-7237.

Yang-Feng, T.L., DeGennaro, L.J. and Francke, U. (1986). Genes for synapsin I, a neuronal phosphoprotein, map to conserved regions of human and murine X chromosomes. *Proc. Natl. Acad. Sci. USA*, **83**, 8679-8683.

Young, R.A., Hagenbuchle, O. and Schibler, U. (1981) Single mouse α -amylase gene specifies two different tissue-specific mRNAs. *Cell*, **23**, 451-458.

Zinn, K., DiMaio, D. and Maniatis, T. (1983) Identification of two distinct regulatory regions adjacent to the human β -interferon gene. *Cell*, **34**, 865-879.

LEBENS LAUF

Name: Carola Anneliese Haas

14.4.58 Geboren in Augsburg

1964 - 1968 Besuch der Grundschule in Kempten/Allgäu

1968 - 1977 Besuch des Carl-von-Linde Gymnasiums in Kempten

1977 Allgemeine Hochschulreife

1977 - 1983 Studium der Biologie an der Universität Ulm

1979 Diplomvorprüfung

1982 Diplomhauptprüfung

1982 - 1983 Diplomarbeit mit dem Thema: " Zelluläre und subzelluläre Localisation von Neuropeptiden im Nervensystem von Aplysia californica"

1983 Diplom in Biologie

1984 - 1987 Promotion in Biologie in der Abt. Neurochemie des Max-Planck-Instituts für Psychiatrie in Martinsried

